THE EFFECTS OF OTR4120 A HEPARAN SULFATE GLYCOSAMINOGLYCAN MIMETIC ON IMPROVING ACUTE AND IMPAIRED WOUND HEALING IN RATS

MIAO TONG

This thesis was funded by a grant from Nuts Ohra Foundation, the Netherlands, grant no. SNO-T-0-0501-159.

Publication of this thesis was financially supported by the Department of Plastic and Reconstructive Surgery of the Erasmus MC, Erasmus University Rotterdam, OTR3 (Organ, Tissue, Regeneration, Repair and Replacement), Perimed AB, and J.E. Jurriaanse Stichting.

Cover photo:

Blue and White Porcelain Jar

China Yuan Dynasty 1271-1368

Height: 26.7 cm

http://blog.sina.com.cn/wv0218

Cover design:

Miao Tong

Layout:

Jingru Wang

Printing:

Off Page, Amsterdam, the Netherlands

ISBN: 978-94-6182-161-4

THE EFFECTS OF OTR4120 A HEPARAN SULFATE GLYCOSAMINOGLYCAN MIMETIC ON IMPROVING ACUTE AND IMPAIRED WOUND HEALING IN RATS

De effecten van OTR4120, een heparansulfaat glycosaminoglycaan-analoog, op het verbeteren van de acute en de verstoorde wondgenezing in de rat

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 31 oktober 2012 om 11.30 uur

door

Miao Tong
Geboren te Nanjing, P.R. China

PROMOTIECOMMISSIE

Promotor:

Prof.dr. S.E.R. Hovius

Overige leden:

Prof.dr. E. Middelkoop

Prof.dr. P. van Zuijlen

Prof.dr. J. F. Lange

Copromotor:

Dr. J.W. van Neck

Paranimfen:

Linda Spiegelberg

Bastiaan Tuk

Dedicated to my parents, my husband Shufan,
daughter Xiaman and son Xiaan

谨以此博士论文献给我的父母，丈夫，
和我亲爱的曼曼和安安

TABLE OF CONTENTS

CHAPTER 1

Introduction

WOUNDS: AN OLD BUT A GROWING CONCERN

Dating back to the prehistoric times, wounds have been common with mankind. The treatment of wounds is an art as old as humanity [1-2]. Today, wounds are of increasing concern in our society in terms of their prevalence and costs. In the developed countries, patients suffering from chronic wounds, such as venous, diabetic, and pressure ulcers, comprise approximately 1% to 2% of the population [3-4]. The incidence of pressure ulcers is as high as 11% in hospitalized patients and as high as 24% in nursing home residents [5]. Diabetic ulcers are recently estimated to occur in 15% of global diabetes population and precede 84% of all diabetes-related lower-limb amputations [6-7]. Moreover, the incidence of ulcer recurrence after complete healing approaches 30% within 1 year [2].

In addition to chronic wounds, there are a large number of acute wounds such as surgical and traumatic wounds. Acute wounds can fail to heal in a timely manner and can also heal with a hypertrophic scar or keloid [8-10].

Wounds and their treatment represent an enormous burden to patients, health care professionals, and the health care system. In the United States, the costs related to chronic wounds are estimated over $25 billion a year [8, 11]. In the European Union, the costs related to pressure and venous ulcers are estimated around €20 billion a year. The burden created by chronic wounds is growing rapidly due to the increasing health care costs, the aging population, and the sharp rise in the incidence of diabetes and obesity [8, 12]. The immense economic and social impact of wounds calls for intensive studies to understand the biology of wound healing and to develop strategies to maximize healing efficiency.

ACUTE WOUND HEALING: A DYNAMIC AND SELF-LIMITING PROCESS

Wound healing is a complex and dynamic process. It requires a large number of interrelated biological events, which are orchestrated over a temporal sequence in response to injury and the

changing wound microenvironment. Wound healing is classically divided into three overlapping and self-limiting phases: inflammation, proliferation, and tissue remodeling [13-16].

Immediately after tissue injury, platelets aggregate to form a fibrin clot. This clot provides a provisional matrix over and through which cells can adhere and migrate. Furthermore, the fibrin clot serves as a reservoir of chemokines, cytokines, and growth factors [17-18]. This cocktail of various soluble factors recruits inflammatory cells to the wound site and initiates the inflammatory phase [19-20].

In the **inflammatory phase**, neutrophils, followed by macrophages, migrate into the wound site to breakdown and remove bacteria and debris. Neutrophils and macrophages also release inflammatory factors, cytokines, chemokines and growth factors that trigger the migration and division of cells involved in the proliferative phase [21-24].

When the inflammatory phase is declining, the **proliferative phase** is underway. In the proliferative phase, the fibrin clot is replaced by granulation tissue and sealed by re-epithelialization. Granulation tissue is formed with highly vascular tissue containing many different cell types and a provisional extracellular matrix (ECM) [25-28]. In this phase, the ECM is constantly synthesized and degraded. The fine-tuned balance between the ECM production and degradation is tightly controlled by many soluble factors such as transforming growth factor beta (TGF-β) [29], matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) [30].

As soon as ECM production and ECM degradation reach equilibrium, the **tissue-remodeling phase** starts. The main feature of this phase is the deposition of collagens in an organized network. During this phase all short-term events, which are activated after injury gradually cease. Most macrophages, endothelial cells, fibroblasts, and myofibroblasts sustain apoptosis or exit from the wound area. They leave a mass that consists mostly of collagens and other matrix proteins. Without an increase in collagen content, the originally disorganized collagen fibers are rearranged, cross-linked, and aligned along tension lines. Type III collagen is prevalent in the proliferative phase. It is gradually degraded and replaced by type I collagen [15, 31]. In this

phase, most of the newly formed blood vessels undergo apoptosis and the wound area develops into an avascular and acellular scar. The strength of the scar never regains the original strength of normal skin again. At the maximal strength for example, a healed wound is 80% as strong as the surrounding normal skin [31].

FEEDFORWARD/FEEDBACK LOOPS: A CONCEPT IN ACUTE WOUND HEALING

An important concept while understanding acute wound healing is the concept of feedforward and feedback loops (Figure 1). During acute wound healing, the progressive healing phase is kicked off by the feedforward signals delivered by the previous phase. The feedforward signals mark the entrance into a transition period where both adjacent processes (i.e. previous and progressive phases) take effect simultaneously. When a progressive phase reaches a critical point (i.e. when a progressive phase is no longer dependent on the feedforward signals from the previous phase to continue), the feedback signals are sent to inhibit or turn off the previous phase. This marks the end of the transition period. The progressive phase progresses and initiates its next phase when it reaches a critical point [32].

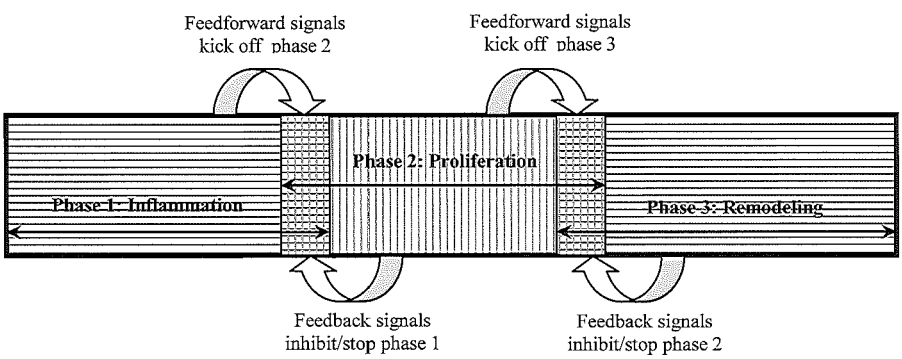

Figure 1. A schematic representation of acute wound healing process with the three overlapping and self-limiting phases under interlocked feedforward/feedback loops.

These interlocked feedforward/feedback loops enable an orderly and coordinated progression of wound healing and prevent any phase from inhibiting and/or perpetuating. If any interlocked loops are broken, the healing process will be enmeshed in a phase (e.g. a self-perpetuating

inflammatory cycle) and unable to progress through the next healing phase. If so, an acute wound can become a chronic wound [32].

CHRONIC WOUND HEALING: AN ARRESTED DISEQUILIBRIUM REPAIR

In contrast to acute wound healing, chronic wound healing fails to progress in an orderly and timely manner to produce anatomic and functional integrity of the injured site [32-34]. The chronic healing process may be expressed as either under-healing (e.g. ulceration), or as over-healing (e.g. hypertrophic and keloid scar formation) [35-36].

Chronic wounds are rarely seen in individuals who are otherwise healthy. In fact, chronic wound patients frequently suffer from co-morbidity such as diabetes and obesity [8]. Multiple factors are associated with chronic wound healing. These factors are categorized into local and systemic factors. Local factors directly influence the wound characteristics. Systemic factors concern the overall health or disease state of an individual that affect his or her ability to heal (Table 1). However, these factors are often interrelated, therefore, their influences are not mutually exclusive [37]. The most common factors are age, diabetes, infection, ischemia, pressure, and venous insufficiency. The most common chronic wound types include venous, diabetic, and pressure ulcers. Together, they constitute approximately 70% of all chronic wounds [34].

Local Factors	Systemic Factors
Infection	Age
Ischemia	Diseases: diabetes, artery disease, peripheral vascular disease
Pressure	Obesity
Venous insufficiency	Immunocompromised conditions: AIDS, radiation therapy, chemotherapy
Desiccation	Congenital healing disorders: epidermolysis bullosa, Ehlers-Danlos syndrome, Marfan's syndrome
Necrosis	
Tissue maceration	Alcoholism
Foreign bodies	Smoking
Trauma	Stress
Edema	Distant cancers
Local cancers	Uremia
Radiation	Nutritional deficiencies
Toxins	Sex hormones
Iatrogenic factors	

Table 1. Factors that are associated with chronic wound healing.

The differences in pathophysiology between acute and chronic wounds are extensive (Table 2) [32, 38]. Excessive inflammation, impaired angiogenesis, abnormalities ECM, and disrupted cell-ECM interactions are major features of chronic wounds [32, 39-40].

Acute wounds (sequential orderly repair)	Chronic wounds (arrested disequilibrium repair)
• Clot formation	• No clot formation
• Activated angiogenesis	• Impaired angiogenesis
• Responsive fibroblasts	• Dysregulated fibroblasts with senescence
• Self-limiting inflammation	• Prolonged inflammation
• Normal MMP/TIMP ratio	• Increased MMP/TIMP ratio
• Net ECM deposition	• ECM degradation
• Normal ECM-cell interactions	• Impaired ECM-cell interactions
• Advancing edge of wound	• Non-advancing/undermined edge of wound
• Migrating keratinocytes	• Non-migrating keratinocytes
• Responsive cells	• Nonresponsive cells
• Normality in ECM	• Abnormalities in ECM
• Balanced proteases and their inhibitor	• Abnormal protease activities
• Bacterial balance	• Bacterial colonization/infection
• Minimal scar formation	• Excessive scar formation

Table 2. Pathophysiological differences between acute and chronic wounds [38].

Chronic wounds exhibit prolonged inflammation that is characterized by abundant neutrophil and macrophage infiltration [41-42]. This persisting infiltration of neutrophils and macrophages plays a major role in generating a protease-rich and pro-oxidant hostile microenvironment [43-46]. Proteolytic degradation products, generated by high protease activity in the chronic wound environment, may exert inhibitory effects on cell functions that are crucial in wound repair [47]. Recent data show that bacterial factors (e.g. toxins and proteases) exert detrimental effects on cell functions (e.g. migration of endothelial cells) [48-50]. In addition, the highly proteolytic and pro-oxidant environment also contributes to the degradation of the ECM components [51-52], growth factors and their receptors [43, 53-54]. These deleterious factors that contribute to the chronic wound environment become promising targets for wound treatment modalities. Potentially, out-of-control inflammation and abnormality of cell-ECM interactions provide therapeutic targets for chronic wound treatment [32, 40].

It's necessary to point out that although chronic wounds are intrinsically different from acute wounds, most chronic wounds do have the self-healing capability.

WOUND TREATMENT: WOUND BED PREPARATION AND HEALING STIMULATION

Wound treatment is to facilitate the acute wound healing process or to convert the chronic wound healing into acute healing by clearing the inhibitors that prevent the healing from going into the next phase, and stimulating the natural wound healing mechanisms.

Wound treatment strategy generally involves wound bed preparation and wound healing stimulation approaches. Wound bed preparation aims to optimize conditions at the wound bed to facilitate the natural wound healing process [55-56]. It can be performed through debridement [57-59], moisture balance [60-61], bacterial control [62-64], exudate management [65], and tissue substitutes for wound healing [11, 66-67]. With the advances in biotechnology and a more detailed understanding of the mechanisms controlling the wound healing process, a great variety of wound stimulation modalities, such as growth factor therapy [68-69], gene therapy [70-71], and stem cell therapy [72-73] have been developed (Table 3). However, a properly performed wound bed preparation is a prerequisite for any wound healing stimulation approaches. In essence the wound environment is a key concern when we are considering the treatment strategies to trigger the self-healing capability of wound tissue.

THE WOUND TREATMENT STRATEGY IN THIS THESIS

The wound treatment strategy in this thesis is to potentiate the wound tissue self-healing capability (i.e. natural wound healing mechanisms) by regulating wound environment through improving the function of the ECM in wounds. The unfolded natural wound healing mechanisms may shift wound repair to wound regeneration that is the aim of wound treatment.

This strategy was verified by using a heparan sulfate glycosaminoglycan (HS-GAG) mimetic, OTR4120 (discussed in detail below).

Treatment	Benefit to wound healing
Debridement (e.g. surgical, enzymatic, chemical debridement)	Bring the wound edges into viable tissue in order to allow cells to deposit the ECM needed for their migration and differentiation, and also promote to release of growth factors
Moisture management	Create an environment that assists in propagating cell migration into the wound area
Exudate management (e.g. by the application of foams, sponges or vacuum therapy)	Drain the wound fluid to clear the ECM degrading enzymes and toxins secreted by bacteria
Local infection management	Clear the wounds from bacteria and the secreted harmful substances and create a bacterial balance
Inflammation management	Reduce the severity and duration of the immune reaction that limit the production of growth factors. Minimize scar formation.
Growth factor therapy	Replenish the wound tissue with growth factors to stimulate wound healing
Tissue engineered skin substitutes	Replace all or some components that make up normal skin (e.g. epidermis and/or dermis, cells and matrix) and induce the expression of growth factors and cytokines that contribute to wound healing
Cell-based therapy (e.g. stem cell therapy)	Introduce cells into wounds and stimulate wound healing
Gene therapy	Target and optimise the delivery of growth factors and cytokines to maximize the therapeutic efficiency

Table 3: Some of the wound treatment modalities and their benefits to wound healing.

THE ECM AND ECM-GROWTH FACTOR INTERACTIONS IN WOUND HEALING

The ECM plays an important role in tissue repair and regeneration. It is the major component of the dermal skin layer. Dynamic interactions between the ECM and growth factors are essential to reestablishing the function of the ECM during wound healing [38]. These interactions mainly take two forms (i.e. the ECM binding of growth factors and integrin-mediated interaction). The

ECM can directly bind to and release growth factors (e.g. HS-GAG binding to growth factors), which serves to protect growth factors from degradation, to promote growth factor receptors activation, and enhances their bioavailability. The ECM can also indirectly adhere to cells via integrins, which enables cells to respond to growth factors and can induce growth factor expression (e.g. adherence of monocytes to the ECM stimulates synthesis of platelet-derived growth factor). In return, growth factors, such as TGF-β, can regulate the ECM by increasing the production of the ECM components or enhancing synthesis of matrix degrading enzymes. Thus, the interactions between growth factors and the ECM are bidirectional [40]. All together, they play a role in maintaining the function of the ECM. In this thesis, the ECM binding of growth factors was focused.

The ECM comprises various families of macromolecules that together form the structural scaffold of tissue and also carry out distinct biological activities [74]. The primary components of ECM are fibrous structural proteins (e.g. collagen and elastin), specialized proteins (e.g. growth factors, and small integrin-binding glycoproteins) and ground substances (i.e. GAGs) [75].

GAGs are linear polysaccharides consisting of various disaccharide-repeating units. HS-GAG is an important member of the GAG family [26, 76]. HS-GAG consists of β2-4 linked uronic acid and glucosamine and encompasses varying degrees of sulfonation [77-79]. HS-GAG is a ubiquitous component of the ECM as many of the matrix proteins, such as collagen, fibronectin, and laminin; possess HS-binding sites [77]. Therefore, in addition to its structural role, HS-GAG has a number of signaling functions through its HS-chains that bind growth factors. This way, HS-GAG can act as a reservoir of growth factors from which growth factors can be sequestered if required [80].

A large variety of proteins can bind HS-GAG. Amongst these are most cell surface proteins, ECM proteins, growth factors, cytokines, chemokines, and morphogens. HS-GAG protects these proteins and molecules from degradation and secures their presence in the ECM. Due to the ubiquitous nature of HS-GAG, the large amount of proteins HS-GAG sequesters, and its fine-tuning effect in growth factor bioavailability, HS-GAG participates in many physiological activities (e.g. cell proliferation, migration, differentiation, cell-cell interactions, and cell-ECM

interactions). This way, HS-GAG plays an important role in maintaining or reestablishing the function of the ECM [80-83].

In summary, HS-GAG can interfere with growth factors either by direct binding growth factors and promoting their receptors or by indirectly controlling their availability and activity through acting as a reservoir from which growth factors can be rapidly released if required.

However, in wound healing, particularly in chronic wounds, the ECM including HS-GAG can be degraded [84]. Injury to tissue initiates the onset of inflammation that releases glycanases, proteases, and MMPs [21, 86-88]. These enzymes destroy the ECM including the HS-GAG [32, 89]. As a result, the orchestrating role of HS-GAG in growth factor and cytokine sequestration is lost. The defective ECM and the disrupted interactions between the ECM and growth factors represent an important characteristic of impaired wound healing [32, 38, 84]. To replace the degraded HS-GAG and thus to reposition growth factors back to the ECM can be a strategy of wound treatment [74, 85]. In this thesis, a HS-GAG mimetic, OTR4120, has been chosen to implement such a strategy.

OTR4120: A HS-GAG MIMETIC

OTR4120 working concept

OTR4120 can replace the destroyed HS-GAG and takes its role in protecting and sequestering growth factors during wound healing. OTR4120 binds to the heparin-binding sites on matrix proteins that become available following HS-GAG degradation to reposition heparin-binding growth factors back to the ECM, protect them from degradation, and activate their receptors. OTR4120 also acts as a reservoir from which growth factors can be rapidly released if required. In this way, OTR4120 improves the bioavailability of growth factors and thus facilitates the interactions between the ECM and growth factors. As a result, the function of the ECM can be regulated and improved. The functioning ECM in wounds contributes to the versatile response of cells and tissues to a changing wound environment and also allows wound tissue to unfold its self-healing capability (i.e. natural wound healing mechanisms) likely to trigger tissue regeneration.

OTR4120 synthesis and structure

OTR4120 is synthesized from dextran T40 by carboxymethylation followed by O-sulfonation (Figure 2). The degrees of substitution of carboxymethyl and sulfate groups are 0.50 and 1.30 respectively. The degree of substitution is defined as the number of substituted groups by glucosidic unit of carboxymethyl and sulfate groups [90-94].

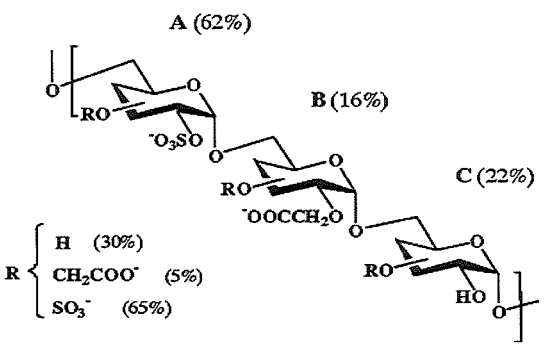

Figure 2. Schematic structure of OTR4120. The dextran derivative on the 1-6 glucose polymeric chain contains carboxymethyl (–CH_2COO–) and sulfate residues (–SO_3–). Three differently substituted glucosidic units are represented according to the nature of the group linked to the C2 position. For easier interpretation, these units were arranged in an arbitrary combination. R represents the possible substituted groups in the global C3 and C4 positions [93].

Differences between OTR4120 and HS-GAG

OTR4120 is structurally similar to the natural HS-GAG. However, a major difference is the stability of OTR4120 in an enzymatic environment [90] and its low anticoagulant activity [92, 95]

The enzyme-resistance of OTR4120 is related to the coupling of the subunits internal bond of the molecules. The β2-4 carbon-carbon binding of the glucosidic units of HS-GAG is prone to enzymatic cleavage whereas the α1-6 carbon-carbon binding of the glucosidic units of OTR4120 is resistant to cleavage by endoglycanases (i.e. heparanase, chondroitinase, hyaluronidase, and dextranase) [90].

OTR4120 displays anticoagulant activity in a dose-dependent manner. The anticoagulant activity of OTR4120 is absent with doses lower than 55 mg/kg [95]. The dose of OTR4120 treatment is in the range of 1–2 mg/kg, therefore, OTR4120 is void of anticoagulant activity [92].

OTR4120 pharmacokinetics

In a pharmacokinetic study following an intravenous (i.v.) injection of OTR4120 at a dose of 5 mg/kg, or an intraperitoneal (i.p.) injection at a dose of 50 mg/kg, or an oral administration at a dose of 70 mg/kg, the distribution was 0.95 L/kg after i.v. injection and i.p. injection, and 4.68 L/kg after oral administration. Plasma clearance was 45, 520 and 514 mL/h/kg after i.v. injection, i.p. injection, and oral administration, respectively. Half-life was 80 min after i.p. injection and 383 min after oral administration [94].

OTR4120 dosage and timing

OTR4120 acts in a bell-shaped dose dependent manner with the optimal dose of 1-2 mg/kg weekly for systemic administration [96-103]. OTR4120 is not effective in the doses over 15 mg/kg [97]. As the number of free HS-binding sites is limited in wound tissue, the excess OTR4120 may compete with the combination of the matrix-OTR4120-growth factors for the HS-binding sites on the matrix.

OTR4120 experimental study

OTR4120 is known to enhance tissue repair in several animal models. They include peripheral nerve injury model in rats [99], necrotic skin ulcer model in mice [103], and secondary degree burns in rats [102, 104].

OTR4120 clinical study

The first clinical pilot study of OTR4120 topical application was reported to improve the healing of severe corneal ulcers [105]. In a recent within-subject study, OTR4120 was demonstrated to reduce wound size and pain perception on pressure and venous ulcers [106]. Van Neck et al. recently described a case study of the complete healing of radiation-induced scalp ulcers after OTR4120 treatment [107]. Although these studies showed the beneficial effects of OTR4120 on healing chronic wounds in patients, they were small-scale trials. Multi-center randomized-controlled clinical trials are needed to obtain conclusive evidence.

THINKING LINE OF THIS THESIS

Chronic wound treatment is difficult. It may because:

- Wound healing is a dynamic process.
- This process is a complex nonlinear system. There are many feedforward/feedback loops, interdependencies, and redundancies in this system network.
- Co-morbidity influences the healing process.

An ideal wound treatment is healing-phase adapted and personalized. It may be feasible, if:

- Wound self-healing capability is potentiated.
- Afterwards /simultaneously exogenous stimuli were applied to stimulate wound healing.

Our strategy is to:

- Improve the function of the ECM in wound environment to facilitate the wound self-healing capability (i.e. natural healing mechanisms).
- The unfolded natural healing mechanisms may shift wound repair towards regeneration, which is a key solution to both under-healing and over-healing problems.

This strategy may be achieved by using HS-GAG mimetic OTR4120. Because it:

- Replaces the destroyed HS-GAG and binds growth factors back to the ECM.
- Protects growth factors from degradation and enhances their bioavailability.
- Acts as a reservoir from which growth factors can be rapidly released if required.
- Restores the ECM-growth factor interactions and thus improves the function of the ECM.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to verify the effects of OTR4120 on both acute and impaired wound healing and to unravel the underlying mechanisms and the clinical implications of OTR4120 in wound treatment.

Chapter 2 and **Chapter 3**: The verification of the effects of OTR4120 started on a surgical full-thickness excisional wound model. This acute wound model clearly represents the three-phase (i.e. inflammation, proliferation and remodeling) acute healing process, by which the potential efficacy of OTR4120 was stringently evaluated. The clinically esteemed outcome measures were first evaluated. They included wound breaking strength: a wound healing end-stage relevant parameter, and vasodilatory capability: a real-time microcirculation parameter (**Chapter 2**). With respect to the three phases of wound healing, inflammation, reepithelialization, epidermal proliferation, granulation tissue formation, angiogenesis, neovascularization, and collagen synthesis and maturation were evaluated to get an overall picture of the effects of OTR4120 on wound healing (**Chapter 3**).

Chapter 4: The promising results of effects of OTR4120 on acute wound healing encouraged us to further investigate the effects of OTR4120 on impaired wound healing. Since the pressure ulcer is one of the major forms of chronic wounds, a clinically relevant pressure ulcer model, induced by ischemia-reperfusion (I-R) injury, was selected. The prophylactic and therapeutic effects of OTR4120 on ulceration and ulcer healing were evaluated in this impaired wound-healing model with the similar wound-relevant parameters that were measured on acute wound model.

Chapter 5: Diabetes-impaired wounds and their treatment are highly clinically concerned. The efficacy of OTR4120 treatment was verified in this chapter using the same I-R-injury-induced pressure ulcer model but created on the streptozotocin-induced diabetic rats. In addition to the wound-relevant parameters that were measured in acute wound and normal pressure ulcer models, the biosynthesis of collagen types and the gene transcription of a number of growth

factors were investigated to reveal the underlying mechanisms of the action of OTR4120 in diabetes-impaired wound healing.

Chapter 6: This chapter discusses the main results obtained in this thesis, draws conclusions and elaborates on future perspectives.

REFERENCES

1. Forrest, R.D., Early history of wound treatment. J R Soc Med, 1982. 75(3): p. 198-205.
2. Robson, M.C., D.L. Steed, and M.G. Franz, Wound healing: biologic features and approaches to maximize healing trajectories. Curr Probl Surg, 2001. 38(2): p. 72-140.
3. Gottrup, F., A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. Am J Surg, 2004. 187(5A): p. 38S-43S.
4. Pieper, B., et al., Wound prevalence, types, and treatments in home care. Adv Wound Care, 1999. 12(3): p. 117-26.
5. Patterson, J.A. and R.G. Bennett, Prevention and treatment of pressure sores. J Am Geriatr Soc, 1995. 43(8): p. 919-27.
6. Shaw, J.E., R.A. Sicree, and P.Z. Zimmet, Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract, 2010. 87(1): p. 4-14.
7. Reiber, G.E., et al., Causal pathways for incident lower-extremity ulcers in patients with diabetes from two settings. Diabetes Care, 1999. 22(1): p. 157-62.
8. Sen, C.K., et al., Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair Regen, 2009. 17(6): p. 763-71.
9. Moro, M.L., et al., Rates of surgical-site infection: an international comparison. Infect Control Hosp Epidemiol, 2005. 26(5): p. 442-8.
10. Franz, M.G., et al., Guidelines to aid healing of acute wounds by decreasing impediments of healing. Wound Repair Regen, 2008. 16(6): p. 723-48.
11. Fan, K., et al., State of the art in topical wound-healing products. Plast Reconstr Surg, 2011. 127 Suppl 1: p. 44S-59S.
12. Natarajan, S., et al., Advances in wound care and healing technology. Am J Clint Dermatol, 2000. 1(5): p. 269-75.
13. Broughton, G., 2nd, J.E. Janis, and C.E. Attinger, The basic science of wound healing. Plast Reconstr Surg, 2006. 117(7 Suppl): p. 12S-34S.
14. Singer, A.J. and R.A. Clark, Cutaneous wound healing. N Engl J Med, 1999. 341(10): p. 738-46.
15. Gurtner, G.C., et al., Wound repair and regeneration. Nature, 2008. 453(7193): p. 314-21.
16. Ramasastry, S.S., Acute wounds. Clin Plast Surg, 2005. 32(2): p. 195-208.
17. Nurden, A.T., et al., Platelets and wound healing. Front Biosci, 2008. 13: p. 3532-48.
18. Laurens, N., P. Koolwijk, and M.P. de Maat, Fibrin structure and wound healing. J Thromb Haemost, 2006. 4(5): p. 932-9.
19. Barrientos, S., et al., Growth factors and cytokines in wound healing. Wound Repair Regen, 2008. 16(5): p. 585-601.
20. Bahou, W.F. and D.V. Gnatenko, Platelet transcriptome: the application of microarray analysis to platelets. Semin Thromb Hemost, 2004. 30(4): p. 473-84.

21. Henry, G. and W.L. Garner, Inflammatory mediators in wound healing. Surg Clin North Am, 2003. 83(3): p. 483-507.
22. Witte, M.B. and A. Barbul, General principles of wound healing. Surg Clin North Am, 1997. 77(3): p. 509-28.
23. Lawrence, W.T. and R.F. Diegelmann, Growth factors in wound healing. Clinics in Dermatology, 1994. 12(1): p. 157-169.
24. Hubner, G., et al., Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. Cytokine, 1996. 8(7): p. 548-56.
25. Adamson, R., Role of macrophages in normal wound healing: an overview. J Wound Care, 2009. 18(8): p. 349-51.
26. Midwood, K.S., L.V. Williams, and J.E. Schwarzbauer, Tissue repair and the dynamics of the extracellular matrix. Int J Biochem Cell Biol, 2004. 36(6): p. 1031-7.
27. Paladini, R.D., et al., Onset of re-epithelialization after skin injury correlates with a reorganization of keratin filaments in wound edge keratinocytes: defining a potential role for keratin 16. J Cell Biol, 1996. 132(3): p. 381-97.
28. Baum, C.L. and C.J. Arpey, Normal cutaneous wound healing: clinical correlation with cellular and molecular events. Dermatol Surg, 2005. 31(6): p. 674-86; discussion 686.
29. Blobe, G.C., W.P. Schiemann, and H.F. Lodish, Role of transforming growth factor beta in human disease. N Engl J Med, 2000. 342(18): p. 1350-8.
30. Borden, P. and R.A. Heller, Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. Crit Rev Eukaryot Gene Expr, 1997. 7(1-2): p. 159-78.
31. Madden, J.W. and E.E. Peacock, Jr., Studies on the biology of collagen during wound healing. I. Rate of collagen synthesis and deposition in cutaneous wounds of the rat. Surgery, 1968. 64(1): p. 288-94.
32. Menke, N.B., et al., Impaired wound healing. Clin Dermatol, 2007. 25(1): p. 19-25.
33. Lazarus, G.S., et al., Definitions and guidelines for assessment of wounds and evaluation of healing. Wound Repair Regen, 1994. 2(3): p. 165-70.
34. Eaglstein, W.H. and V. Falanga, Chronic wounds. Surg Clin North Am, 1997. 77(3): p. 689-700.
35. O'Leary, R., E.J. Wood, and P.J. Guillou, Pathological scarring: strategic interventions. Eur J Surg, 2002. 168(10): p. 523-34.
36. Satish, L. and S. Kathju, Cellular and Molecular Characteristics of Scarless versus Fibrotic Wound Healing. Dermatol Res Pract, 2010. 2010: p. 790234.
37. Guo, S. and L.A. Dipietro, Factors affecting wound healing. J Dent Res, 2010. 89(3): p. 219-29.
38. Schultz, G.S. and A. Wysocki, Interactions between extracellular matrix and growth factors in wound healing. Wound Repair Regen, 2009. 17(2): p. 153-62.
39. Eming, S.A., et al., Regulation of angiogenesis: Wound healing as a model. Progress in Histochemistry and Cytochemistry, 2007. 42(3): p. 115-170.

40. Gregory S. Schultz, A.W., Interactions between extracellular matrix and growth factors in wound healing. Wound Repair and Regeneration, 2009. 17(2): p. 153-162.
41. Diegelmann, R.F., Excessive neutrophils characterize chronic pressure ulcers. Wound Repair Regen, 2003. 11(6): p. 490-5.
42. Diegelmann, R.F. and M.C. Evans, Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci, 2004. 9: p. 283-9.
43. Yager, D.R., et al., Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids. J Invest Dermatol, 1996. 107(5): p. 743-8.
44. Lobmann, R., et al., Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. Diabetologia, 2002. 45(7): p. 1011-6.
45. Ashcroft, G.S., et al., Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. Nat Med, 2000. 6(10): p. 1147-53.
46. Wenk, J., et al., Selective pick-up of increased iron by deferoxamine-coupled cellulose abrogates the iron-driven induction of matrix-degrading metalloproteinase 1 and lipid peroxidation in human dermal fibroblasts in vitro: a new dressing concept. J Invest Dermatol, 2001. 116(6): p. 833-9.
47. Hoffman, R., S. Starkey, and J. Coad, Wound fluid from venous leg ulcers degrades plasminogen and reduces plasmin generation by keratinocytes. J Invest Dermatol, 1998. 111(6): p. 1140-4.
48. Athanasopoulos, A.N., et al., The extracellular adherence protein (Eap) of Staphylococcus aureus inhibits wound healing by interfering with host defense and repair mechanisms. Blood, 2006. 107(7): p. 2720-7.
49. Aepfelbacher, M., et al., Bacterial toxins block endothelial wound repair. Evidence that Rho GTPases control cytoskeletal rearrangements in migrating endothelial cells. Arterioscler Thromb Vasc Biol, 1997. 17(9): p. 1623-9.
50. Bowler, P.G., Wound pathophysiology, infection and therapeutic options. Ann Med, 2002. 34(6): p. 419-27.
51. Grinnell, F., C.H. Ho, and A. Wysocki, Degradation of fibronectin and vitronectin in chronic wound fluid: analysis by cell blotting, immunoblotting, and cell adhesion assays. J Invest Dermatol, 1992. 98(4): p. 410-6.
52. Grinnell, F. and M. Zhu, Fibronectin degradation in chronic wounds depends on the relative levels of elastase, alpha1-proteinase inhibitor, and alpha2-macroglobulin. J Invest Dermatol, 1996. 106(2): p. 335-41.
53. Lauer, G., et al., Generation of a novel proteolysis resistant vascular endothelial growth factor165 variant by a site-directed mutation at the plasmin sensitive cleavage site. FEBS Lett, 2002. 531(2): p. 309-13.
54. Lauer, G., et al., Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. J Invest Dermatol, 2000. 115(1): p. 12-8.
55. Falanga, V., Classifications for wound bed preparation and stimulation of chronic wounds. Wound Repair Regen, 2000. 8(5): p. 347-52.

56. Schultz, G., et al., Wound healing and TIME; new concepts and scientific applications. Wound Repair Regen, 2005. 13(4 Suppl): p. S1-11.
57. Hess, C.T. and R.S. Kirsner, Orchestrating wound healing: assessing and preparing the wound bed. Adv Skin Wound Care, 2003. 16(5): p. 246-57; quiz 258-9.
58. Lloyd, J.R. and D.W. Hight, Early laminar excision: improved control of burn wound sepsis by partial dermatome debridement. J Pediatr Surg, 1978. 13(6D): p. 698-706.
59. Attinger, C.E., E. Bulan, and P.A. Blume, Surgical debridement. The key to successful wound healing and reconstruction. Clin Podiatr Med Surg, 2000. 17(4): p. 599-630.
60. Korting, H.C., C. Schollmann, and R.J. White, Management of minor acute cutaneous wounds: importance of wound healing in a moist environment. J Eur Acad Dermatol Venereol, 2011. 25(2): p. 130-7.
61. Okan, D., et al., The role of moisture balance in wound healing. Adv Skin Wound Care, 2007. 20(1): p. 39-53; quiz 53-5.
62. Nathan, C., Points of control in inflammation. Nature, 2002. 420(6917): p. 846-52.
63. Wright, J.B., et al., Efficacy of topical silver against fungal burn wound pathogens. Am J Infect Control, 1999. 27(4): p. 344-50.
64. Fung, H.B., J.Y. Chang, and S. Kuczynski, A practical guide to the treatment of complicated skin and soft tissue infections. Drugs, 2003. 63(14): p. 1459-80.
65. Vowden, K. and P. Vowden, Understanding exudate management and the role of exudate in the healing process. Br J Community Nurs, 2003. 8(11 Suppl): p. 4-13.
66. Rizzi, S.C., et al., Recent advances in dermal wound healing: biomedical device approaches. Expert Rev Med Devices, 2010. 7(1): p. 143-54.
67. Marston, W.A., et al., The efficacy and safety of Dermagraft in improving the healing of chronic diabetic foot ulcers: results of a prospective randomized trial. Diabetes Care, 2003. 26(6): p. 1701-5.
68. Fu, X., et al., Engineered growth factors and cutaneous wound healing: success and possible questions in the past 10 years. Wound Repair Regen, 2005. 13(2): p. 122-30.
69. Mustoe, T.A., et al., A phase II study to evaluate recombinant platelet-derived growth factor-BB in the treatment of stage 3 and 4 pressure ulcers. Arch Surg, 1994. 129(2): p. 213-9.
70. Branski, L.K., et al., Gene therapy in wound healing: present status and future directions. Gene Ther, 2007. 14(1): p. 1-10.
71. Eming, S.A., T. Krieg, and J.M. Davidson, Gene therapy and wound healing. Clin Dermatol, 2007. 25(1): p. 79-92.
72. Sellheyer, K. and D. Krahl, Skin mesenchymal stem cells: prospects for clinical dermatology. J Am Acad Dermatol, 2010. 63(5): p. 859-65.
73. Wu, Y., R.C. Zhao, and E.E. Tredget, Concise review: bone marrow-derived stem/progenitor cells in cutaneous repair and regeneration. Stem Cells, 2010. 28(5): p. 905-15.
74. Agren, M.S. and M. Werthen, The extracellular matrix in wound healing: a closer look at therapeutics for chronic wounds. Int J Low Extrem Wounds, 2007. 6(2): p. 82-97.

75. Daley, W.P., S.B. Peters, and M. Larsen, Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci, 2008. 121(Pt 3): p. 255-64.
76. Taylor, K.R. and R.L. Gallo, Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. FASEB J, 2006. 20(1): p. 9-22.
77. Dreyfuss, J.L., et al., Heparan sulfate proteoglycans: structure, protein interactions and cell signaling. An Acad Bras Cienc, 2009. 81(3): p. 409-29.
78. Bernfield, M., et al., Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem, 1999. 68: p. 729-77.
79. Tumova, S., A. Woods, and J.R. Couchman, Heparan sulfate proteoglycans on the cell surface: versatile coordinators of cellular functions. Int J Biochem Cell Biol, 2000. 32(3): p. 269-88.
80. Flaumenhaft, R. and D.B. Rifkin, Extracellular matrix regulation of growth factor and protease activity. Curr Opin Cell Biol, 1991. 3(5): p. 817-23.
81. Watt, F.M. and H. Fujiwara, Cell-extracellular matrix interactions in normal and diseased skin. Cold Spring Harb Perspect Biol, 2011. 3(4).
82. Hynes, R.O., The extracellular matrix: not just pretty fibrils. Science, 2009. 326(5957): p. 1216-9.
83. Eckes, B., R. Nischt, and T. Krieg, Cell-matrix interactions in dermal repair and scarring. Fibrogenesis Tissue Repair, 2010. 3: p. 4.
84. Cook, H., et al., Defective extracellular matrix reorganization by chronic wound fibroblasts is associated with alterations in TIMP-1, TIMP-2, and MMP-2 activity. J Invest Dermatol, 2000. 115(2): p. 225-33.
85. Gandhi, N.S. and R.L. Mancera, Heparin/heparan sulphate-based drugs. Drug Discov Today, 2010. 15(23-24): p. 1058-69.
86. Nwomeh, B.C., et al., Dynamics of the matrix metalloproteinases MMP-1 and MMP-8 in acute open human dermal wounds. Wound Repair Regen, 1998. 6(2): p. 127-34.
87. Barrick, B., E.J. Campbell, and C.A. Owen, Leukocyte proteinases in wound healing: roles in physiologic and pathologic processes. Wound Repair Regen, 1999. 7(6): p. 410-22.
88. Bame, K.J., Heparanases: endoglycosidases that degrade heparan sulfate proteoglycans. Glycobiology, 2001. 11(6): p. 91R-98R.
89. Eming, S.A., T. Krieg, and J.M. Davidson, Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol, 2007. 127(3): p. 514-25.
90. Ikeda, Y., et al., Synthesis and biological activities of a library of glycosaminoglycans mimetic oligosaccharides. Biomaterials, 2011. 32(3): p. 769-76.
91. Rouet, V., et al., A synthetic glycosaminoglycan mimetic binds vascular endothelial growth factor and modulates angiogenesis. J Biol Chem, 2005. 280(38): p. 32792-800.
92. Charef, S., et al., Toxicological evaluation of RGTA OTR4120, a heparan sulfate mimetic. Food Chem Toxicol, 2010. 48(7): p. 1965-8.

93. Barbosa, I., et al., A synthetic glycosaminoglycan mimetic (RGTA) modifies natural glycosaminoglycan species during myogenesis 10.1242/jcs.01607. J Cell Sci, 2005. 118(1): p. 253-264.
94. Charef, S., D. Papy-Garcia, and J. Courty, Absorption and tissue distribution of a novel carboxymethyldextran after oral administration. Biomed Pharmacother, 2010. 64(9): p. 627-32.
95. Charef, S., et al., Effects on coagulation of a synthetic heparan mimetic given intraperitoneally or orally. J Biomed Mater Res A, 2007. 83(4): p. 1024-31.
96. Tong, M., et al., Stimulated neovascularization, inflammation resolution and collagen maturation in healing rat cutaneous wounds by a heparan sulfate glycosaminoglycan mimetic, OTR4120. Wound Repair Regen, 2009. 17(6): p. 840-52.
97. Escartin, Q., et al., A new approach to treat tissue destruction in periodontitis with chemically modified dextran polymers. Faseb J, 2003. 17(6): p. 644-51.
98. Meddahi, A., et al., Pharmacological studies of RGTA(11), a heparan sulfate mimetic polymer, efficient on muscle regeneration. J Biomed Mater Res, 2002. 62(4): p. 525-31.
99. Zuijdendorp, H.M., et al., Significant reduction in neural adhesions after administration of the regenerating agent OTR4120, a synthetic glycosaminoglycan mimetic, after peripheral nerve injury in rats. J Neurosurg, 2008. 109(5): p. 967-73.
100. Tong, M., et al., RGTA OTR 4120, a heparan sulfate proteoglycan mimetic, increases wound breaking strength and vasodilatory capability in healing rat full-thickness excisional wounds. Wound Repair Regen, 2008. 16(2): p. 294-9.
101. Tong, M., et al., Heparan sulfate glycosaminoglycan mimetic improves pressure ulcer healing in a rat model of cutaneous ischemia-reperfusion injury. Wound Repair Regen, 2011. 19(4): p. 505-14.
102. Garcia-Filipe, S., et al., RGTA OTR4120, a heparan sulfate mimetic, is a possible long-term active agent to heal burned skin. J Biomed Mater Res A, 2007. 80(1): p. 75-84.
103. Barbier-Chassefiere, V., et al., Matrix therapy in regenerative medicine, a new approach to chronic wound healing. J Biomed Mater Res A, 2009. 90(3): p. 641-7.
104. Zakine, G., et al., Matrix therapy with RGTA OTR4120 improves healing time and quality in hairless rats with deep second-degree burns. Plast Reconstr Surg, 2011. 127(2): p. 541-50.
105. Chebbi, C.K., et al., [Pilot study of a new matrix therapy agent (RGTA OTR4120) in treatment-resistant corneal ulcers and corneal dystrophy. J Fr Ophtalmol, 2008. 31(5): p. 465-71.
106. Groah, S.L., et al., Regenerating matrix-based therapy for chronic wound healing: a prospective within-subject pilot study. Int Wound J, 2011. 8(1): p. 85-95.
107. Van Neck, J.W., et al., Unexpected healing of radiation-induced scalp lesions with OTR4120, a heparan sulfate mimetic. Eur J Dermatol, 2011. 21(3): p. 451-2.

CHAPTER 2

OTR4120

a heparan sulfate glycosaminoglycan mimetic increases wound breaking strength and vasodilatory capability in healing rat full-thickness excisional wound

Miao Tong, Mariken M. Zbinden, Ineke M. Hekking, Marcel Vermeij, Denis Barritault, Johan W. van Neck

Wound Repair Regen 2008; 16: 294-299

ABSTRACT

ReGeneraTing Agents (RGTAs), a family of polymers engineered to protect and stabilize heparin-binding growth factors, have been shown to promote tissue repair and regeneration. In this study, the effects of one of these polymers, OTR4120, on healing of full-thickness excisional wounds in rats were investigated. Two 1.5 cm diameter circular full-thickness excisional wounds were created on the dorsum of a rat. After creation of the wounds, OTR4120 was applied. The progress of healing was assessed quantitatively by evaluating the wound closure rate, vasodilatory capability, and wound breaking strength. The results showed a triple increase of the local vascular response to heat provocation in the OTR4120-treated wounds as compared to vehicle- treated wounds. On days 14 and 79 after surgery, the wounds treated with OTR4120 gained skin strength 12 and 48 percent of the unwounded skin, respectively, and displayed a significantly increased gain in skin strength when compared to control animals. These results raise the possibility of efficacy of OTR4120 in accelerating surgically cutaneous wound healing by enhancing the wound breaking strength and improving the microcirculation.

INTRODUCTION

Wound healing is a dynamic and complex process of tissue repair, which involves a number of cellular and molecular events. These events include cell migration, proliferation, angiogenesis, and extracellular matrix (ECM) deposition and degradation [1]. Many of these cellular activities are regulated by growth factors, especially heparin-binding growth factors (HBGFs), such as fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β) [2-4]. HBGFs are stored and protected by specific interactions with the heparan sulfates, a group of glycosaminoglycans highly organized in the ECM. As a natural component of the ECM, heparan sulfates play a key role in chemical signaling between cells by binding and regulating the activities of HBGFs, proteolytic enzymes, and protease inhibitors [5]. However, after injury, heparan sulfate glycosaminoglycans will be degraded by locally secreted matrix metalloproteases, serine proteases and heparinases [6]. Consequently, the balanced interplay of HBGFs released to surrounding tissue will be disturbed. Prolonged absence of a proper functioning ECM may lead to a chronic wound healing state. In an attempt to replace the degraded heparan sulfates at the wound site, dextran polymers have been modified with defined amounts of substituted carboxymethyl (CM), carboxymethyl sulfate (CMS), carboxymethyl benzylamide (CMB). They are named RGTAs, for ReGeneraTing Agents. In wound bed, RGTAs bind the free heparin-binding sites of the matrix proteins, and mimic the stabilizing and protecting properties of heparan sulfates towards HBGFs. This way RGTAs enhance the bioavailability of these HBGFs [7-11]. In addition to their HBGFs protecting and stabilizing properties, RGTAs have been found to inhibit enzymatic activities of human neutrophil elastase [12] and plasmin [13-14].

In vivo, RGTAs have been shown to promote tissue repair in several experimental models including bone [15-16], muscle [11,17], colon [18-19], and burned skin [20]. However, little is known about the potential efficacies of RGTAs on surgical acute wounds, which are the common wounds in clinics. Therefore, the objective of the current investigation was to evaluate the effects of one of these RGTAs, OTR4120, on surgical wound healing using a rat full-

thickness excisional wound model. The wound contraction, breaking strength and reactive hyperaemia following heat provocation were assessed.

MATERIALS AND METHODS

WAG/RijHsd male rats (30, 8 to 11 weeks old, 200 to 250 g) were purchased from Harlan (Zeist, the Netherlands). They were housed three animals per cage prior to surgery. Rats were exposed to a 12-hour light/dark cycle and fed with a standard laboratory diet (Hope Farms, Woerden, the Netherlands) with food and water available ad libitum. The rats were allowed to acclimatize to their environment for one week prior to the procedure. The experimental protocol was approved by the Animal Experiments Committee under the national experiments on animals act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.

Full-thickness excisional wound model

Rats were randomly separated into three experimental groups of 10 each to serve 3 experimental end-points (i.e. days 14, 21, and 79 after wounding). In each group, 5 rats were randomly selected for RGTA OTR4120 treatments (i.e. RGTA-treated group) and the other 5 rats were for vehicle (i.e. physiological salt solution) application (i.e. control group).

Rats were anesthetized using isoflurane (Isoflurane, Rhodia Organique Fine Ltd., Bristol, UK) in oxygen as carrier. The dorsal surface was shaved with an electric clipper and then rinsed with alcohol gauze. Two dermal circles (15 mm in diameter with centers 3 cm apart and 4 cm caudal to the scapulae) were measured and marked by a skin marker. Excisions extending through the panniculus carnosus were cut using a pair of surgical scissors. Bleeding was stopped by compression with sterile cotton sticks. All animals received an intramuscular (i.m.) injection of the analgesic, Temgesic™, (Rekitt Benckiser Healthcare, Ltd., Hull, East Yorkshire, UK) at a dose of 0.05mg/kg on days 0, 1 and 2 after surgery. Wounds were left undressed and all rats were housed individually in elevated stainless steel-bottom cages for the first 2 days after surgery. Afterwards, they were transferred to the normal bedding cages.

OTR4120 treatments

The OTR4120 was obtained from the Organ, Tissue, Regeneration, Repair and Replacement (OTR3, Paris, France) and prepared as previously described [20]. Briefly, this water-soluble dextran derivative was prepared from T40 dextran by carboxymethylation with monochloracetic acid treatment, followed by O-sulfonation with SO3-DMF complex in the presence of 2-methyl-2-butene. Degrees of substitution, defined as the number of groups substituted by glucosidic units, were 0.50 for carboxymethyl groups and 1.30 for sulfate groups.

For OTR4120 treatment, 30 minutes after wounding (day 0), a single dose of 80μL OTR4120 at a concentration of 0.1mg/mL in sterile physiological salt solution (B. Braun Melsungen AG, Germany) was topically applied to the wounds (4.5 μg OTR4120/cm^2 wound). OTR4120 was also systematically applied by i.m. injection at a dose of 1mg/kg bodyweight on day 0, thereafter, weekly at the first month and once every two weeks until the end of experiment. The control groups received an equal amount of physiological salt solution in an identical protocol. Experimenters were blinded to the treatments.

Wound closure rate measurements

Digital photographs of the wounds with a ruler as reference were taken on days 7, 14, and 21 after wounding using a Samsung Digimax A5 (Samsung, Kyungki-Do, Korea). Wound areas were analyzed by tracing the wound margins and calculating the pixel areas using the public domain image processing and analysis program, ImageJ 1.37 (National Institutes of Health, Bethesda, MD). Wound closure rate was calculated according to the following formula:

$$\text{Wound closure rate } (\%) = (\text{Area}_{day0} - \text{Area}_{dayn}) / \text{Area}_{day0} \times 100\ \%$$

Where Area_{day0} is the initial wound area at day 0 and Area_{dayn} is the area at day n after wounding.

Vasodilatory capability measurements

Using Laser Doppler flowmetry (LDF) measurements with local heat provocation, the vasodilatory capabilities of both wounded and normal skin with or without OTR4120 treatment were monitored on days 13, 20 and 78 after wounding. Measuring was not begun until a steady respiration was obtained. The area of the skin of measurement was initially warmed to 33 °C using a 1cm-diameter Thermostatic Laser Doppler Probe (PROBE 457, Perimed AB, Stockholm, Sweden), a combined laser Doppler and thermostatic probe used for local heat provocation while blood perfusion is measured. The optical fibers are integrated in the heated area and thus the whole skin area under the probe will be heated. The skin under the probe was kept on 33 °C until a stable baseline was established for 5 minutes. Subsequently the skin was heated to 44 °C and maintained to this temperature throughout the 10 minutes heating period. The microcirculatory response to this local heating was monitored using a laser Doppler perfusion monitor (LDPM, Perimed Periflux System 5000, Perimed AB) with the thermostatic PROBE 457. Continuous LDPM employed coherent laser light at a wavelength of 780 nm. LDF was measured in arbitrary perfusion unit (PU). Sampled LDF was interfaced to a personal computer through an analog-to-digital converter using PeriSoft data-acquisition software (PeriSoft for Windows Version 2.5, Perimed AB). The PU value was calculated as mean PU on the plateau. The reactive hyperaemia rate, which was also expressed percentage change from baseline, was calculated for data analysis according to the following formula:

Reactive hyperaemia rate (%) = % change from baseline = $[(PU_{44}\,^{\circ}C - PU_{33}\,^{\circ}C) / PU_{33}\,^{\circ}C] \times 100$ %

Where $PU_{44}\,^{\circ}C$ is the PU at 44 °C and $PU_{33}\,^{\circ}C$ is the PU at 33 °C, which is the baseline of the measurement.

Breaking strength measurements

On days 14, 21 and 79 after wounding, all the rats from each group were sacrificed. Breaking strength was measured by a modification of the method by Ko et al. [21]. Briefly, the entire

dorsal pelt, containing both wounded areas, was immediately excised and uniformly cut into standard dumbbell-shaped strips with a central width 4 mm, and a length 45 mm, using a custom-built punch (the Medical Instrumental Department, Erasmus MC, University Medical Center, Rotterdam, the Netherlands). The wound side was positioned midway down the length of the strip. Three strips were sampled from each animal: two strips from each wounded side and one strip from the normal skin in between the two wounds. Strips were kept moist by wrapping them in gauzes soaked with ice-cold physiological salt solution and were measured immediately after harvesting.

Breaking strengths were measured using a Testometric® AX, M250-2.5KN tensiometer (Testometric Company Ltd., Lancashire, UK). The dumbbell-shaped strips were fixed perpendicularly between the two clips of the tensiometer and subjected to a constant strain rate of 60mm/minute using a 1.0 kg force transducer. Breaking strength was recorded as the maximum load measured before skin failure and expressed in Newtons (N). The wound breaking strength ratio was calculated for data analysis according to the following formula:

Wound breaking strength ratio = wounded skin breaking strength (N) / normal skin breaking strength (N)

Statistical analysis

Because two wounds were created on each rat, we used mixed model ANOVA with wound breaking strength ratio or reactive hyperaemia rate as the dependent variable and time and treatment as the independent factors. No structure was imposed on the 2 x 2 covariance matrix of the residuals. p-value ≤ 0.10 was considered to demonstrate a statistically significant time-by-treatment interaction. Statistical analyses were performed with commercially available SPSS for windows (version 11). Results were considered statistically significant at $p \leq 0.05$. All quantitative data are presented as mean values ± standard deviation (SD).

RESULTS

OTR4120 effects on wound contraction

Circular full-thickness excisional wounds were created on the dorsum of rats. For each wound the percentage of wound closure was calculated at timed intervals by measuring the unclosed wound area and expressed as percentage of the original wound area. By the first week, the wound closure rate reaches approximately 30%. The time to complete wound closure was on the end of the third week after wounding. No statistically significant differences between OTR4120 treated and control groups were observed in wound closure rate at all time points (data not shown).

OTR4120 effects on vascular response to local heating

The superficial vascular capacities of the tissue in wounds and also the normal skin in between the two wounds were monitored using LDF with local heat provocation. The percentages of increased blood flow perfusion over baseline flow, following the local heat provocation, were 27 ± 21%, 100 ± 80%, and 202 ± 21% in the wounded skin of OTR4120-treated rats on days 13, 20, and 78 after wounding, respectively. In the wounds of control rats, the changes of vascular response were 15 ± 9%, 37 ± 20%, and 62 ± 11% on days 13, 20, and 78 after wounding, respectively. It showed that RGTA OTR4120 treatment significantly increased the vasodilatory capability of wounded skin on both day 20 ($p < 0.01$) and day 78 after wounding ($p < 0.001$) when compared to their controls. The normal skin in between the two wounds didn't show significantly increased blood flow to heat provocation after OTR4120 treatments at all time points (Figure 1).

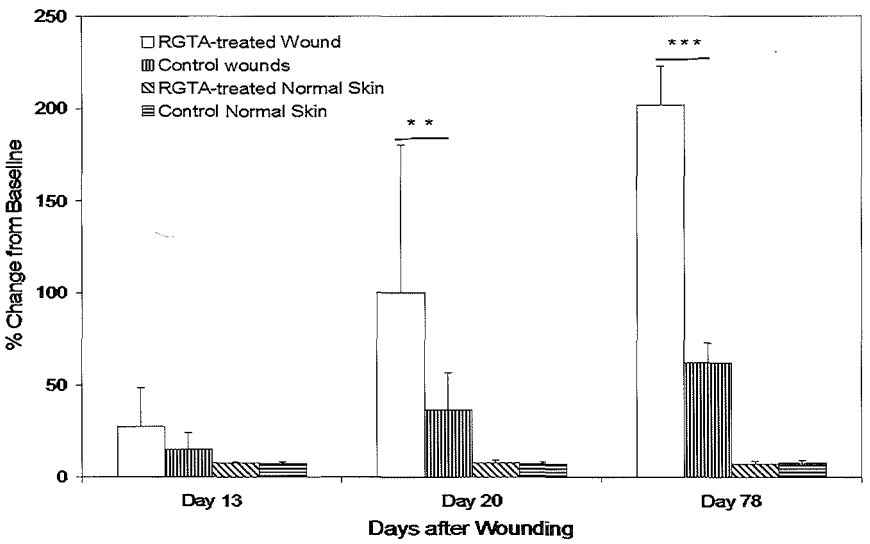

Figure 1. The wounded skin and normal skin vascular responses to local heating (44°C for 10 minutes), expressed as the percentages of the increase blood flow over baseline flow over baseline flow, measured by Laser Doppler flowmetry with local heat provocation on days 13, 20, and 78 after wounding. Error bars represent the standard deviation. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate the significant differences between treated groups and control groups.

OTR4120 effects on wound breaking strength

The breaking strength ratios OTR4120 treated groups, when compared to their respective controls, were 0.12 ± 0.04 vs. 0.07±0.03 on day 14 after wounding; 0.14 ± 0.04 vs. 0.10 ± 0.04 on day 21 after wounding; and 0.48 ± 0.13 vs. 0.39 ± 0.05 on day 79 after wounding, respectively. Statistical differences between OTR4120 treated groups and their controls were found on day 14 ($p < 0.05$) and day 79 ($p < 0.001$) (Figure 2).

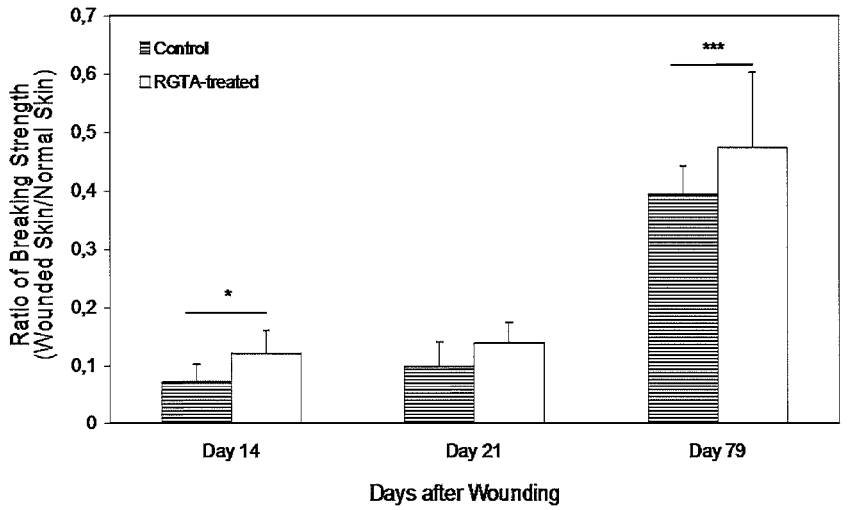

Figure 2. Wound breaking strength measured as the ratio of the breaking strength of the wounded skin versus that of normal skin on days 14, 21, and 79 after wounding. Error bars represent the standard deviation. $*p < 0.05$ and $***p < 0.001$ indicate the significant differences between treated groups and control groups.

DISCUSSION

RGTAs are a family of chemically modified dextran polymers engineered to mimic the properties of heparan sulfate towards HBGFs. Under physiological conditions, these growth factors are naturally stored in the heparan sufhate moieties of the ECM. However, after tissue injury, activated inflammatory cells release glycanases and proteases that degrade heparan sulfates and the ECM components including heparan sulfate. Subsequently, bound growth factors are no longer protected and liberated which makes growth factors susceptible to be degradated. RGTAs are engineered to replace the degraded heparan sulfate at bodily sites of injury. It binds the free heparin-binding sites of ECM proteins that are exposed after heparan sulfate degradation and provide fixation/protecting sites for growth factors. Like other dextran

derivatives, RGTAs are resistant to heparanases. When bound to ECM proteins, RGTAs will protect growth factors from further degradation and inhibit the action of proteases such as plasmin [14, 22], and elastase [23]. The protection of growth factors by RGTAs results in a preservation of the natural endogenous signalling of tissue and is reflected by improvement of tissue repair and regeneration [10, 13, 14, 24].

In this study, we demonstrated that the heparan sulfate mimetic OTR4120, a member of RGTA family, improves surgical full-thickness wound healing, assessed through wound skin breaking strength and vasodilatory capability to local heating provocation.

Estimation of microcirculation function is valuable in the analysis of the events that occur during wound repair. In this study, cutaneous blood flow and vascular response to local heat provocation have employed to assess the microvasculation. This was measured by LDF with local heat provocation, which is commonly used as a non-invasive technique for monitoring continuous microvascular and endothelial function in a number of different organs and under a variety of different conditions [25-28]. This method allows measurement of the capability of the vasculature to dilate in response to heating, which is related not only to the number of capillaries in the skin tissue but also the endothelial function [29]. The results of this study showed a persistent lesion capillary blood flow increase, as induced by heating, in OTR4120-treated wounds. It demonstrates that OTR4120 treatment improves the quantity of microcirculation during wound healing. The possible mechanism could be due to the fact that OTR4120 modulates and accelerates and angiogenesis [30]. It was reported that OTR4120 had high affinity binding to VEGF165 as compared with heparin, and potentiated the affinity of VEGF165 for VEGF receptor-1 and -2 and for neuropilin-1. OTR4120 also potentiated VEGF165-induced proliferation and migration of human umbilical vein endothelial cells [30]. The increased capillary growth delivers more oxygen and nutrients to wound bed, leading to prompted synthesis and deposition of newly vascularized connective tissue. This, in turn, may contribute to the improved wounds. To be noticed, these temperature response effects were seen only in wounded skin. We also measured the blood flow and its response to local heat provocation of the normal skin in between the two wounds. Normal skin did not show increased blood flow significantly to heat provocation after OTR4120 treatment compared to control

animals. This implies that OTR4120 may not compete and replace the natural endogenous heparan sulfate that bound on the matrix proteins. OTR4120 may only bind the free heparin-binding sites of the matrix proteins when heparan sulfate has been degraded following tissue injury. The specific activity of RGTA, solely at injured sites, was also demonstrated in RGTA11, another member of RGTA family, in a model of ischemic muscle healing using an intravenously injected ^{3}H–RGTA11. RGTA11 was found readily eliminated in a non-injured rat, whereas in animals after muscle crushing, ^{3}H–RGTA11 remained detectable solely in the damaged muscle until regeneration was completed [11]. These evidences of the targeting effect of RGTA to the injury tissue broaden the interest of RGTA in clinical application.

Wound skin biomechanical strength restoration is another vital indicator of wound healing process, especially of end-stage wound healing assessment. The rate at which wounds gain strength is fast, reflecting a higher degree of division and migration of fibroblasts into wound and a faster rate of accumulation of collagens, more importantly, of collagen degradation and remodeling with the formation of larger collagen bundles and increased in the number of intermolecular cross-links [31]. In this study, we demonstrated that OTR4120 treatment results in a significant enhancement of wound breaking strength not only in a short-term (14 days after wounding), but also in a long-term (79 days after wounding) observation period. On day 79, the wounds with OTR4120 treatment gained 48% of their unwounded skin strength, which was significantly higher than that of vehicle-treated wounds (39%). These results indicate that the beneficial effects of OTR4120 treatment may involve the maturation and remodeling of wound tissue. The long-term effect of OTR4120 on improving collagen maturation was also demonstrated in a rat burn model [20]. It was reported that OTR4120 restored an appropriate level of collagen expression, thereby could potentially reduce fibrotic evolution within 6 days and maintaining these beneficial effects up to 10 months after the burn [20]. During the remodeling phase, the last and longest stage of wound repair, the new connective tissue matrixes are organized into a scar. This new connective tissue organization in scar differs from dermis; a parallel arrangement of scar collagen fiber bundles replaces the basket-weave collagen fiber bundles of dermis [32]. The scar formation impedes the normal biochemical strength restoration. It has been reported that even at the end of a year, the rat healed incisional wounds were only about 80 % as strong as unwounded skin [33]. Therefore, the long-term beneficial effects of

OTR4120 on restoration of wound strength also imply that OTR4120 treatment may have a potential on reducing scar formation. Previous studies have shown that RGTA treatment reversed the abnormal collagen production in intestinal biopsies of Crohn's disease patients, where a marked reduction of the collagen III/I ratio was observed [34]. RGTA has also been reported to induce matrix remodeling by decreasing the expression of collagen III in smooth muscle cells [8, 35-36] and burned skin [20]. However, this is a preliminary study. The precise mechanism of action of OTR4120 in enhancing wound breaking strength needs to be further studied.

Overall, we have shown that OTR4120 treatment enhances the rat full-thickness surgical wound healing by improving vasodilatory capability and wound biomechanical strength restoration. This result tempts us to presume further studies on the mechanisms of action of OTR4120 in wound healing.

ACKNOWLEDGMENTS

The authors thank Dr. P.G.H. Mulder from the Department of Epidemiological Biostatistics of Erasmus Medical Centre, Rotterdam, the Netherlands, for the help on statistical analysis. This research was supported by a grant from the Nuts Ohra Foundation, grant No. SNO-T-0-0501-159.

REFERENCES

1. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 1999; 341:(10):738-46.
2. Barnes GL, Kostenuik PJ, Gerstenfeld LC, Einhorn TA. Growth factor regulation of fracture repair. J Bone Miner Res 1999; 14:(11):1805-15.
3. Lawrence WT, Diegelmann RF. Growth factors in wound healing. Clin Dermatol 1994; 12:(1):157-69.
4. Bolander ME. Regulation of fracture repair by growth factors. Proc Soc Exp Biol Med 1992; 200:(2):165-70.
5. Chang Z, Meyer K, Rapraeger AC, Friedl A. Differential ability of heparan sulfate proteoglycans to assemble the fibroblast growth factor receptor complex in situ. Faseb J 2000; 14:(1):137-44.
6. Bame KJ. Heparanases: endoglycosidases that degrade heparan sulfate proteoglycans. Glycobiology 2001; 11:(6):91R-98R.
7. Rapraeger AC, Guimond S, Krufka A, Olwin BB. Regulation by heparan sulfate in fibroblast growth factor signaling. Methods Enzymol 1994; 245:219-40.
8. Mestries P, Borchiellini C, Barbaud C, Duchesnay A, Escartin Q, Barritault D, Caruelle JP, Kern P. Chemically modified dextrans modulate expression of collagen phenotype by cultured smooth muscle cells in relation to the degree of carboxymethyl, benzylamide, and sulfation substitutions. J Biomed Mater Res 1998; 42:(2):286-94.
9. Moscatelli D. Basic fibroblast growth factor (bFGF) dissociates rapidly from heparan sulfates but slowly from receptors. Implications for mechanisms of bFGF release from pericellular matrix. J Biol Chem 1992; 267:(36):25803-9.
10. Rouet V, Meddahi-Pelle A, Miao HQ, Vlodavsky I, Caruelle JP, Barritault D. Heparin-like synthetic polymers, named RGTAs, mimic biological effects of heparin in vitro. J Biomed Mater Res A 2006; 78:(4):792-7.
11. Meddahi A, Bree F, Papy-Garcia D, Gautron J, Barritault D, Caruelle JP. Pharmacological studies of RGTA(11), a heparan sulfate mimetic polymer, efficient on muscle regeneration. J Biomed Mater Res 2002; 62:(4):525-31.
12. Meddahi A, Lemdjabar H, Caruelle JP, Barritault D, Hornebeck W. FGF protection and inhibition of human neutrophil elastase by carboxymethyl benzylamide sulfonate dextran derivatives. Int J Biol Macromol 1996; 18:(1-2):141-5.
13. Ledoux D, Papy-Garcia D, Escartin Q, Sagot MA, Cao Y, Barritault D, Courtois J, Hornebeck W, Caruelle JP. Human plasmin enzymatic activity is inhibited by chemically modified dextrans. J Biol Chem 2000; 275:(38):29383-90.

14. Meddahi A, Lemdjabar H, Caruelle JP, Barritault D, Hornebeck W. Inhibition by dextran derivatives of FGF-2 plasmin-mediated degradation. Biochimie 1995; 77:(9):703-6.
15. Lafont J, Baroukh B, Berdal A, Colombier ML, Barritault D, Caruelle JP, Saffar JL. RGTA11, a new healing agent, triggers developmental events during healing of craniotomy defects in adult rats. Growth Factors 1998; 16:(1):23-38.
16. Albo D, Long C, Jhala N, Atkinson B, Granick MS, Wang T, Meddahi A, Barritault D, Solomon MP. Modulation of cranial bone healing with a heparin-like dextran derivative. J Craniofac Surg 1996; 7:(1):19-22.
17. Zimowska M, Constantin B, Papy-Garcia D, Raymond G, Cognard C, Caruelle JP, Moraczewski J, Martelly I. Novel glycosaminoglycan mimetic (RGTA, RGD120) contributes to enhance skeletal muscle satellite cell fusion by increasing intracellular Ca2+ and calpain activity. J Cell Physiol 2005; 205:(2):237-45.
18. Meddahi A, Alexakis C, Papy D, Caruelle JP, Barritault D. Heparin-like polymer improved healing of gastric and colic ulceration. J Biomed Mater Res 2002; 60:(3):497-501.
19. Meddahi A, Benoit J, Ayoub N, Sezeur A, Barritault D. Heparin-like polymers derived from dextran enhance colonic anastomosis resistance to leakage. J Biomed Mater Res 1996; 31:(3):293-7.
20. S. Garcia-Filipe VB-C, C. Alexakis, E. Huet, D. Ledoux, M. E. Kerros, E. Petit, D. Barritault, J. P. Caruelle, P. Kern,. RGTA OTR4120, a heparan sulfate mimetic, is a possible long-term active agent to heal burned skin. Journal of Biomedical Materials Research Part A 2007; 80A:(1):75-84.
21. Ko J, Ross J, Awad H, Hurwitz H, Klitzman B. The effects of ZD6474, an inhibitor of VEGF signaling, on cutaneous wound healing in mice. J Surg Res 2005; 129:(2):251-9.
22. Ledoux D, Papy-Garcia D, Escartin Q, Sagot M-A, Cao Y, Barritault D, Courtois J, Hornebeck W, Caruelle J-P. Human Plasmin Enzymatic Activity Is Inhibited by Chemically Modified Dextrans. J. Biol. Chem. 2000; 275:(38):29383-90.
23. Meddahi A, Lemdjabar H, Caruelle J-P, Barritault D, Hornebeck W. FGF protection and inhibition of human neutrophil elastase by carboxymethyl benzylamide sulfonate dextran derivatives. International Journal of Biological Macromolecules 1996; 18:(1-2):141-45.
24. Barritault D, Caruelle JP. [Regenerating agents (RGTAs): a new therapeutic approach]. Ann Pharm Fr 2006; 64:(2):135-44.
25. Wilson SB, Jennings PE, Belch JJ. Detection of microvascular impairment in type I diabetics by laser Doppler flowmetry. Clin Physiol 1992; 12:(2):195-208.
26. Veves A, Akbari CM, Primavera J, Donaghue VM, Zacharoulis D, Chrzan JS, DeGirolami U, LoGerfo FW, Freeman R. Endothelial dysfunction and the expression

of endothelial nitric oxide synthetase in diabetic neuropathy, vascular disease, and foot ulceration. Diabetes 1998; 47:(3):457-63.

27. Stewart J, Kohen A, Brouder D, Rahim F, Adler S, Garrick R, Goligorsky MS. Noninvasive interrogation of microvasculature for signs of endothelial dysfunction in patients with chronic renal failure. Am J Physiol Heart Circ Physiol 2004; 287:(6):H2687-96.
28. Boignard A, Salvat-Melis M, Carpentier PH, Minson CT, Grange L, Duc C, Sarrot-Reynauld F, Cracowski JL. Local hyperemia to heating is impaired in secondary Raynaud's phenomenon. Arthritis Res Ther 2005; 7:(5):R1103-12.
29. Gooding KM, Hannemann MM, Tooke JE, Clough GF, Shore AC. Maximum skin hyperaemia induced by local heating: possible mechanisms. J Vasc Res 2006; 43:(3):270-7.
30. Rouet V, Hamma-Kourbali Y, Petit E, Panagopoulou P, Katsoris P, Barritault D, Caruelle JP, Courty J. A synthetic glycosaminoglycan mimetic binds vascular endothelial growth factor and modulates angiogenesis. J Biol Chem 2005; 280:(38):32792-800.
31. Bailey AJ, Bazin S, Sims TJ, Le Lous M, Nicoletis C, Delaunay A. Characterization of the collagen of human hypertrophic and normal scars. Biochim Biophys Acta 1975; 405:(2):412-21.
32. Ehrlich HP, Keefer KA, Myers RL, Passaniti A. Vanadate and the absence of myofibroblasts in wound contraction. Arch Surg 1999; 134:(5):494-501.
33. Levenson SM, Geever EF, Crowley LV, Oates JF, 3rd, Berard CW, Rosen H. The Healing of Rat Skin Wounds. Ann Surg 1965; 161:293-308.
34. Alexakis C, Caruelle JP, Sezeur A, Cosnes J, Gendre JP, Mosnier H, Beaugerie L, Gallot D, Malafosse M, Barritault D, Kern P. Reversal of abnormal collagen production in Crohn's disease intestinal biopsies treated with regenerating agents. Gut 2004; 53:(1):85-90.
35. Alexakis C, Mestries P, Garcia S, Petit E, Barbier V, Papy-Garcia D, Sagot MA, Barritault D, Caruelle JP, Kern P. Structurally different RGTAs modulate collagen-type expression by cultured aortic smooth muscle cells via different pathways involving fibroblast growth factor-2 or transforming growth factor-beta1. Faseb J 2004; 18:(10):1147-9.
36. Alexakis C, Guettoufi A, Mestries P, Strup C, Mathe D, Barbaud C, Barritault D, Caruelle JP, Kern P. Heparan mimetic regulates collagen expression and TGF-beta1 distribution in gamma-irradiated human intestinal smooth muscle cells. Faseb J 2001; 15:(9):1546-54.

CHAPTER 3

Stimulated neovascularization, inflammation resolution, and collagen maturation on healing rat cutaneous wounds by a heparan sulfate glycosaminoglycan mimetic, OTR4120

Miao Tong, Bastiaan Tuk, Ineke M. Hekking, Marcel Vermeij, Denis Barritault, Johan W. van Neck

Wound Repair Regen 2009; 17: 840-852

ABSTRACT

Heparan sulfate glycosaminoglycans (HS-GAGs) are not only the structural elements of tissue architecture, but also regulate the bioavailability and transduction pathways of heparan sulfate-bound polypeptides released by cells or extracellular matrix. Heparan sulfate-bound polypeptides include inflammatory mediators, chemokines, angiogenic factors, morphogens, and growth factors that induce cell migration, proliferation, and differentiation in wound healing. OTR4120, a polymer engineered to mimic the properties of HS-GAGs, is used to replace the natural HS-GAGs that are degraded during wound repair, and enhance the tissue regeneration by preserving the cellular microenvironment and the endogenous signals needed for tissue repair and regeneration. We previously demonstrated that OTR4120 treatment had a long-term effect on increasing wound breaking strength and vasodilation in healing rat full-thickness excisional wounds. The present study investigates the underlying mechanisms of the action of OTR4120 on improving the quality of cutaneous wound repair. We found that OTR4120 treatment stimulated inflammation resolution and increased neovascularization. OTR4120 treatment also promoted epidermal migration and proliferation during reepithelialization. Moreover, the granulation tissue formation and collagen maturation were improved in OTR4120-treated wounds. Three months after wounding, the effects of OTR4120 treatment on vascularization and inflammation resolution were normalized, except for an improved neodermis. We conclude that OTR4120 is a potential matrix therapeutic-agent that ensures the quality of acute cutaneous wound repair and may restore impaired wound healing characterized by deficient angiogenesis and prolonged inflammation.

INTRODUCTION

During wound healing, interactions between the extracellular microenvironment and the cell surface influence cell proliferation, differentiation, adhesion, migration, and tissue shape [1-2]. Heparan sulfate glycosaminoglycans (HS-GAGs), an important family of the extracellular matrix (ECM) components, play a crucial role in these interactions, as they are components of both basement and plasma membranes [3]. The heparan sulfate (HS) chains bind to cell surfaces or ECM proteins, and in this way HS-GAGs provide a framework for matrix architecture, and also implicate cell-cell and cell-matrix signalling and interactions. The HS moiety binds with high affinity a range of cytokines and growth factors, including fibroblast growth factors (FGFs), transforming growth factor β (TGF-β), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [3]. This way, the growth factors that play a pivotal role in wound healing are stabilized and protected [4], and their activities are regulated and potentiated [5-7]. Moreover, the plasma membrane HS-GAGs can form ternary structures with growth factors and their receptors, by doing so modulate the response of receptor-bearing cells to growth-factor induced signalling [3, 7-8]. Through this mechanism, HS-GAGs act as "molecular matchmakers", orchestrating the functional assembly of multi-protein complexes to ensure the function of ECM for the quality of wound healing [9].

Injury to tissue initiates the onset of acute inflammation. The activated inflammatory cells release glycanases (such as heparanases and chondroitinases) and proteases (such as serine proteases and metalloproteases) into the injury site [10-11]. These enzymes aid wound repair by eliminating bacterial contaminations, digesting matrix proteins, releasing growth factors from the ECM, and remodeling the tissue [12]. However, these enzymes can also destroy the ECM when their actions are prolonged, inappropriate, or excessive [12-15]. It has been reported that the level of various serine proteases is elevated in chronic wounds [11, 14-17]. In addition, the expression and the activity of various matrix metalloproteases (MMPs) have been found highly up regulated in chronic wounds [15, 18-20]. As a consequence of this highly proteolytic microenvironment, the matrix proteins and mediators crucial for wound healing become targets of wound glycanases and/or proteases. Indeed, the ECM components and the growth factors pivotal for wound repair, such as VEGF and PDGF, have been shown to be degraded and

inactivated by proteolytic cleavage in chronic wounds [15, 21-23]. Moreover, the degraded products can further promote inflammation and result in a vicious circle of chronic inflammation as well as a self-perpetuating cycle of ECM destruction and reconstruction [12-13]. We hypothesize that exogenously introducing degradation-resistant HS-GAG mimetic into the wounded ECM will halt these self-perpetuating cycles. HS-GAG mimetic will take over the role of degraded HS-GAGs, stabilize, protect, and potentiate the matrix proteins and wound repair mediators and restore the function of the ECM.

OTR4120 is such a protease and glycanase resistant HS-GAG mimetic. It is derived from a glycosidic polymer of dextran T40 and functionalized with carboxylate sulfate groups [24-25]. It is structurally related to natural HS-GAGs and mimics their protecting and potentiating properties toward heparin-binding growth factors (HBGFs), such as FGFs, TGF-β, and VEGF. OTR4120 is known to enhance tissue repair in several animal models, including peripheral nerve injury in rats [26], burned skin in rats [24], and chronic skin ulcers in mice [27]. We recently reported that OTR4120 increased wound breaking strength and vasodilatory capability on healing rat full-thickness excisional wounds [28]. In vitro, OTR4120 was demonstrated to potentiate VEGF165-induced proliferation and migration of human umbilical vein endothelial cells [25]. In a clinical pilot study, OTR4120 was reported to improve the healing of severe corneal ulcers and dystrophy [29]. From these studies, together with the fact that OTR4120 acts as a HS-GAG mimetic, we postulate that, in the wound tissue, OTR4120 can replace the degraded HS-GAGs, and bind to the free heparin-binding sites (which become available after HS-GAGs degradation) of the matrix proteins (such as collagens, laminin, and fibronectin). This way, OTR4120 protects the matrix proteins from further proteolysis, and provides fixations and protecting sides for the newly synthesized as well as the free growth factors released from the action of HS-GAGs degradation during wound healing. This way OTR4120 offers a matrix therapy to restore the natural cellular microenvironment and the endogenous signalling of cell communications needed for tissue regeneration, thereby halting the self-perpetuating cycles in impaired healing wounds [25, 30-31].

In this study, we investigate the effects of OTR4120 treatment on healing rat cutaneous wounds and its underlying mechanism by evaluating inflammation, neovascularization, epidermal proliferation, reepithelialization, and collagen maturation during cutaneous wound repair.

MATERIALS AND METHODS

Animals

WAG/RijHsd male rats (50, 8 to 11 weeks old, 200 to 250 g) purchased from Harlan, Zeist, the Netherlands were exposed to a 12-h light/dark cycle and fed a standard laboratory diet (Hope Farms, Woerden, the Netherlands) with food and water available *ad libitum*. Prior to the procedure, the rats were allowed to acclimatize to their environment for one week. The experimental protocol was approved by the local Animal Experiments Committee (i.e. adherence to the rules prescribed in the national Animals Act, which implements the 'Guidelines on the protection of experimental animals' by the Council of Europe, 1986: Directive 86/609/EC).

OTR4120

OTR4120 was produced by Organ, Tissue, Regeneration, Repair and Replacement (OTR3, Paris, France) as an alpha1-6 carboxymethyl sulphated glucose polymer of average molecular mass of 77kD [24-25]. Lyophilized OTR4120 was rehydrated in a physiological salt solution (0.9 % NaCl, Braun Melsungen AG, Germany) at a concentration of 1.3 µM OTR4120 for topical application and 13 µM OTR4120 for intramuscular injection.

Full-thickness wounds and OTR4120 treatments

Rats were randomly separated into five experimental groups of 10 rats each, to serve five experimental time points (i.e. days 3, 7, 14, 21 and 79 after wounding). In each group, rats were equally and randomly divided into experimental group (OTR4120-treated, n = 5) and control group (physiological salt solution-treated, n = 5).

Two full-thickness wounds of 15-mm in diameter were created on the dorsal skin of a rat as previously described [28]. Thirty minutes after surgery, both wounds on a rat were received a single topical application of OTR4120, with a volume of 80 μL (104 pmol) for each wound. At the same time, rats also received intramuscular injection of OTR4120 on the right mid-thigh at a dose of 13 nmol/kg bodyweight (200 to 250 μL according to the bodyweight) and, thereafter, weekly for the first month and once every two weeks until the end of experiment [24, 26, 28]. As a control, the same volume of physiological salt solution was applied in an identical protocol. Wounds were left uncovered. Experimenters were blinded to the treatments.

Rats were euthanized at each indicated time point. Two hours before euthanasia, the rats from day 7 time-point group were received an intraperitoneal injection of bromodeoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) at a dose of 228 nmoL/kg bodyweight. Immediately after euthanasia, the wound including 2 mm of the surrounding unwounded skin was excised and cut into halves. One half was fixed in 10% phosphate-buffered formalin for histology. The other half of the wound, excising the surrounding unwounded skin, was snap-frozen in liquid nitrogen and kept in -80°C for Western blot analysis and collagen assay.

Histomorphometric analysis of wound reepithelialization and granulation tissue formation

Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E). Using a light microscope with an Olympus eyepiece micrometer (A×0071, 20.4 mm square, Olympus, Tokyo, Japan), reepithelialization was determined by measuring the bilateral lengths of the inward-migrating epithelial tongues from the wound margins to the tips of the epithelial cells. The thickness of the granulation tissue or neodermis was analyzed at the center of the wound by measuring the vertical distance between the surface of the granulation tissue (or the base membrane of the epidermis) and the surface of the subcutis.

Immunohistochemistry

Immunohistochemical localizations of CD68, CD34, VEGF, flt-1 (VEGF-receptor 1), and BrdU were detected using avidin-biotin-peroxidase complex immunohistochemistry. Antigen retrieval was performed in either way: (1) using a heat-mediated method: heating sections in TE buffer

(10 mM Tris, 1 mM EDTA, pH 9.0) containing 0.05% Tween 20 for 20 minutes at 90°C in a water bath, and (2) using an enzymatic method: incubating sections in TE buffer containing 0.1% trypsin (Invitrogen, Carlsbad, CA) for 20 minutes at 37°C. For BrdU staining, DNA was denaturized before antigen retrieval by incubating the sections in 2 N HCL for 30 minutes at 37°C. After antigen retrieval, sections were exposed to 0.1% hydrogen peroxide in phosphate-buffered saline (PBS) containing 0.1% Tween 20 for 15 minutes at 37°C and then incubated in blocking buffer (4% non-fat milk powder in PBS containing 0.1% Tween 20) for 30 minutes. Without washes, sections were incubated with the primary antibodies listed in Table1, respectively, overnight at 4°C and then further incubated with a corresponding biotinylated secondary antibodies (R&D systems, Minneapolis, MN). Detection of the antibody complex was performed with streptavidin-peroxidase (R&D systems) and 3,3'-diaminobenzidine (DAB, Dako, Carpinteria, CA). Finally, sections were counterstained with hematoxylin and mounted with Permount® (Fisher Scientific, Pittsburgh, PA). Immunohistochemistry in the absence of the corresponding primary antibody served as a negative control. Unless specified otherwise, the procedure was performed at room temperature.

Antigen	Species in which the antibody was raised	Marker for	Product no./ Clone no.	Working dilution	Antigen retrieval method	Supplier
CD68	Mouse	Monocytes /macrophages [32]	MCA341R/E D1	1:100	Enzymatic	AbD Serotec
CD34	Goat	Endothelial cells [33]	AF4117	1:200	Enzymatic	R&D Systems
VEGF	Mouse	Angiogenic factors [34]	sc-7269	1:100	Enzymatic	Santa Cruz Biotechnology
flt-1	Rabbit	VEGF receptor-1 [35]	sc-316	1:50	Heat-mediated	Santa Cruz Biotechnology
BrdU	Mouse	Proliferating cells [36]	B8434 /BU-33	1:200	Heat-mediated	Sigma-Aldrich

Table 1. Antibodies used to detect specific antigens in paraffin-embedded sections or in wound tissue homogenates by Western blot analysis.

The number of the proliferating epidermal cells was determined by manually counting the number of BrdU-positive cells in the entire neoepidermis. In order to compare the distribution of

the proliferating epidermal cells on the migrating epidermal tongue and the wound margin, the stained cells in those two areas were also counted separately.

For evaluation of CD68, CD34, VEGF and flt-1 staining, the overview of the positive-signal density was scored semi-quantitatively as 1 (= absent), 2 (= low), 3 (= medium), 4 (= strong), and 5 (= very strong).

Two observers, who were blinded to the treatments, carried out both counting and scoring independently. Counting was accepted as comparable when the difference between the counts for the two observations on the same sections was no more than 10%. After each observer's score had been ranked separately, the samples appearing in the upper and lower 50% of each ranking were compared. If there was an overlap of more than 70% between the two observers the score was accepted. The mean of the counts or scores accepted was calculated for each individual sample.

Western blot analysis

The frozen wound tissue was placed in a liquid nitrogen-cooled vial and pulverized using Braun Mikro-Dismembrator (B. Braun Biotech International, Melsungen, Germany). The powdered tissue was thawed in 20 vol of ice-cooled Laemmli loading buffer (Bio-Rad, Hercules, CA) and centrifuged at 16,000 × g for 30 minutes at 4 °C. The supernatant was collected and the protein concentration was determined using RcDc protein assay kit (Bio-Rad). Equivalent amount of proteins was separated on a SDS polyacrylamide gel, and transferred overnight in a cold room onto a polyvinylidene difluoride membrane (Bio-Rad). Blots were stained reversibly with 2% Ponceau S (Sigma-Aldrich) to confirm the equal protein loading. Then the membrane was incubated in blocking buffer (3 % bovine serum albumin [Sigma-Aldrich] in 10 mM Tris, 150 mM NaCl, containing 0.1% Tween-20, pH 7.6) for 2 hours and then probed with 1:1,000 diluted primary antibodies (i.e. VEGF or flt-1; Table1) for 1 hour, and afterwards, overnight at 4°C. Following washes, the membrane was incubated with the corresponding 1:4000 diluted peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) for 3 hours. Signals were visualized using Supersignal® West Femto Maximum Sensitivity Substrate (Pierce) on a high performance chemiluminescence film (GE Healthcare Europe GmbH, Diegem, Belgium).

Protein expression was determined by the quantitative assessment of the band volume using a calibrated GS-800 scanner (Bio-Rad) with quantity one® 1-D analysis software, version 4.6.3. (Bio-Rad). This assessment was presented by an optical density (OD) multiplied by band area (OD × mm^2), and displayed in an arbitrary unit. Unless specified otherwise, the procedure was performed at room temperature.

Collagen solubility evaluation

The solubility profile of collagen, in the wound tissue harvested at day 14 after wounding, was assessed by sequential extractions of collagen in neutral salt buffer, acetate buffer, and acetate-pepsin buffer as described below. The powdered wound tissue was extracted in neutral salt buffer (0.05 M Tris, 1 M NaCl, pH 7.5) containing 1% (V/V) protease inhibitor cocktail (Sigma-Aldrich) overnight at 4°C. The contents were then centrifuged at 15,000 x *g* for 1 hour at 4°C, after which the supernatants were pooled for salt-soluble collagen (SSC) measurement. The precipitates were suspended in acetate buffer (0.5 M CH3COOH, pH 3) containing 1% (V/V) protease inhibitor cocktail overnight at 4°C, and then centrifuged at 15,000 x *g* for 1 hour at 4°C. The supernatants, containing acid-soluble collagen (ASC), were collected. The precipitates were further extracted in acetate-pepsin buffer (100 mg pepsin [Sigma-Aldrich] per gram powdered tissue in 0.5 M CH3COOH) overnight at 4°C to obtain pepsin-soluble collagen (PSC). The residue that remained after salt, acid, and pepsin extractions was insoluble collagen (ISC). ISC was solubilized in Milli-Q water (Millipore, Billerica, MA) by denaturation of collagen at 80°C for 1 hour in a water bath. The collagen contents in the pooled fractions were determined by Sircol™ Collagen Assay kit (Biocolor, Ltd., County Antrim, BT, UK) according to the manufacturer's protocol. Sircol™ Collagen Assay is a quantitative colorimetric method in which Sirius Red dye binds to the side chains of the amino acids found in collagen. Briefly, 50 µL of the extract was mixed gently with 1 mL of Sircol dye reagent and incubated for 30 minutes. The unbound dye was removed by centrifugation, and the collagen-bound dye was dissolved in 0.5M NaOH. Absorbance was measured at 540 nm using a VersaMaxTM microplate reader (Molecular Devices, Sunnyvale, CA). All measurements were performed in duplicate.

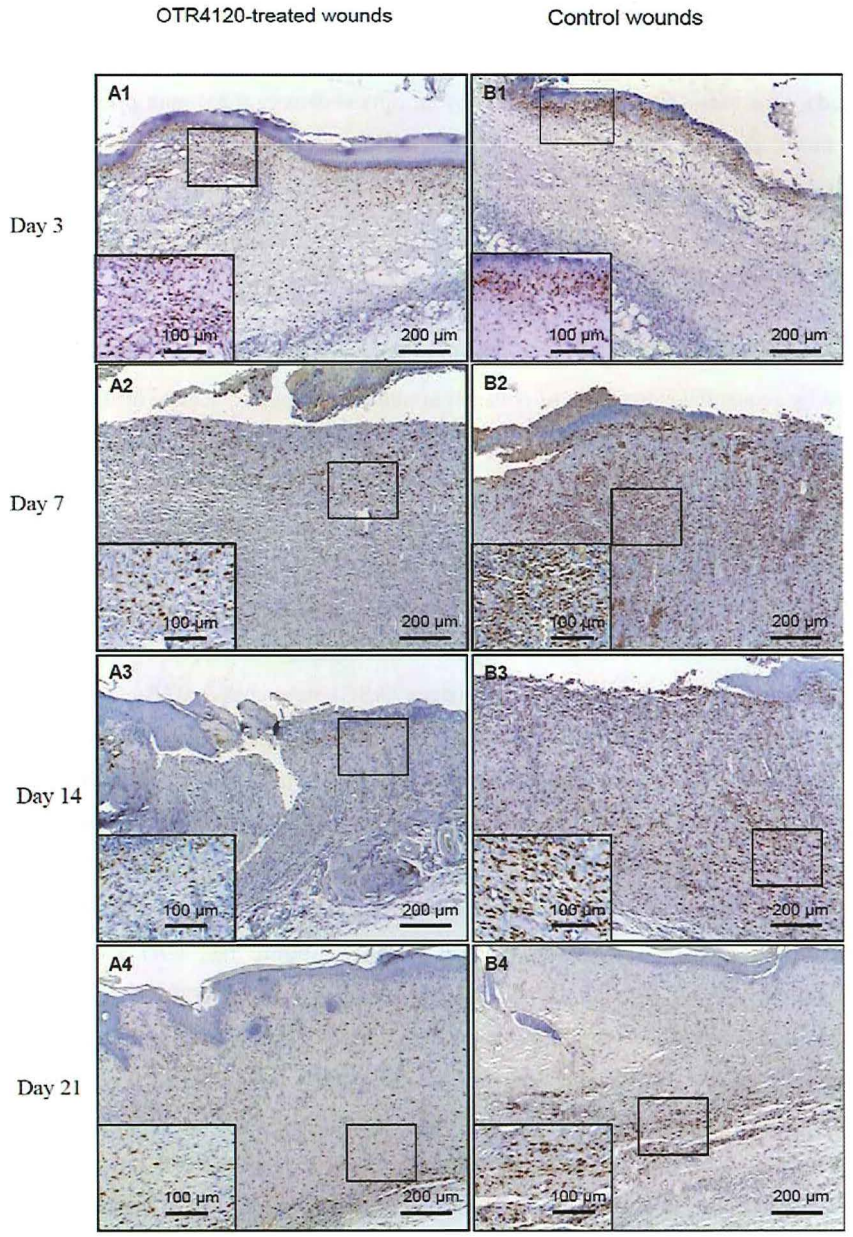

Figure 1. Effect of OTR4120 treatment on the resolution of inflammation assessed by the monocyte/macrophage marker CD68 immunohistochemistry. Representative CD68-staining sections of OTR4120-treated wounds (A1-4) and control wounds (B1-4) at days 3, 7, 14, and 21 after wounding, respectively. CD68 staining started reducing in the OTR4120-treated wounds at day 7 and persisted for 2 weeks (at days 14 and 21). Original magnification × 40. Original inset magnification × 200.

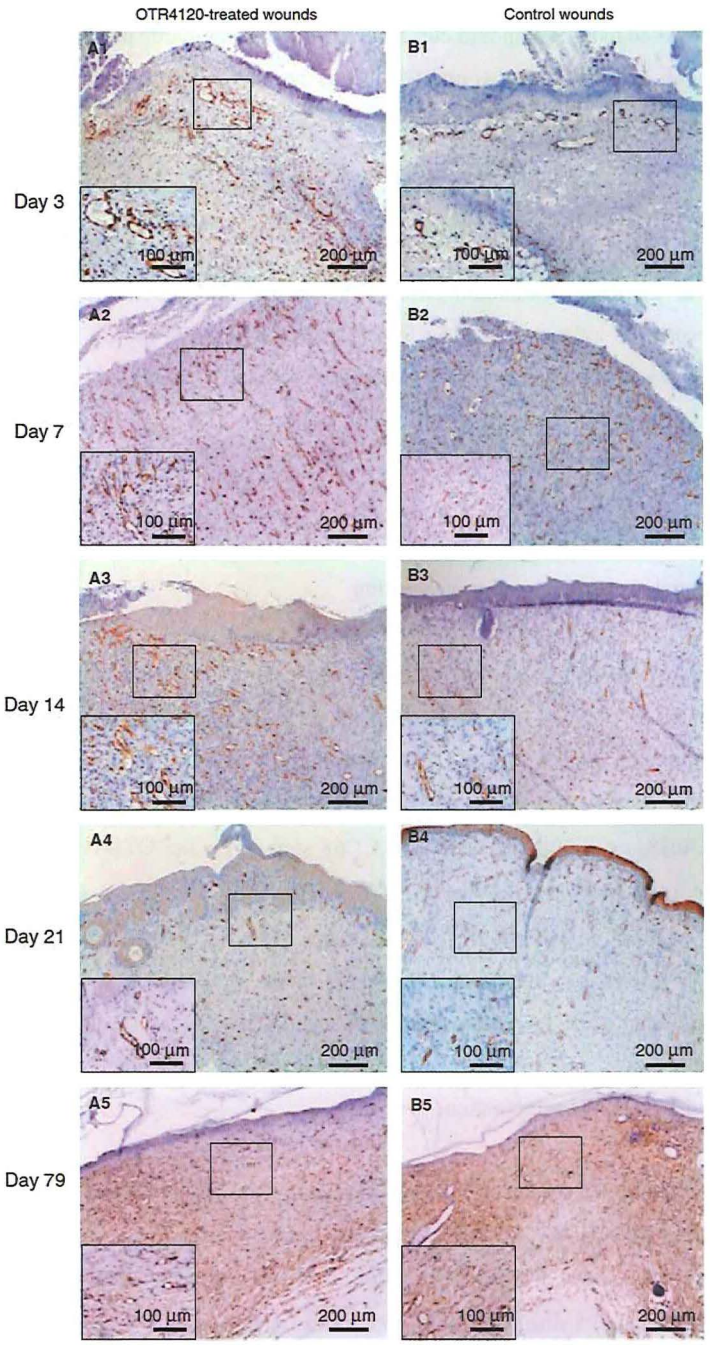

Figure 2. Effect of OTR4120 treatment on neovascularization assessed by the endothelial cell marker CD34 immunohistochemistry. Representative CD34-staining sections of OTR4120-treated wounds (A1-5) and control wounds (B1-5) at days 3, 7, 14, 21, and 79 after wounding, respectively. OTR4120-treated wounds had a higher density of CD34 staining at days 3, 7, and 14 than of the control wounds, respectively. Original magnification × 40. Original inset magnification × 200.

Concentrations were calculated using a standard curve generated with standards provided by the manufacturer. The collagen content in the wound tissue was normalized to weight per mg of powered tissue. Unless specified otherwise, the procedure was performed at room temperature.

Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were conducted using two-tailed independent t-test for comparisons between groups. Statistical difference was defined as $p \leq 0.05$. Analyses were performed with SPSS, version 14 (Chicago, IL).

RESULTS

OTR4120 treatment stimulates inflammation resolution

Inflammation was evaluated by detecting monocytes/macrophages in wounds using an anti-CD68 antibody (Figure 1). At day 3, no significant difference of the CD68-staining score between OTR4120-treated and control wounds was found (3.05 ± 0.19 vs. 3.22 ± 0.39). However, at day 7, CD68 staining was significantly reduced in the OTR4120-treated wounds (3.31 ± 0.07 vs. 4.49 ± 0.18, $p< 0.001$). The reduced CD68 staining in the OTR4120-treated wounds was continually observed at days 14 and 21 (2.76 ± 0.30 vs. 3.69 ± 0.22, at day 14, $p< 0.05$; and 2.40 ± 0.24 vs. 3.42 ± 0.23, at day 21, $p< 0.05$). This indicates that OTR4120 treatment accelerates inflammation resolution, however, does not influence the early monocyte/macrophage response during the inflammatory phase.

OTR4120 treatment induces neovascularization

Neovascularization was visualized by immunostaining of endothelial cells in the wound tissue using the endothelial cell marker CD34. OTR4120 treatment accelerated neovascularization in the first 2 weeks after wounding (days 3, 7, and 14; Figure 2). At day 3, the CD34-staining score of OTR4120-treated wounds was 1.8 times as high as that of the control wounds (3.96 ± 0.38 vs.

2.16 ± 0.29, $p< 0.01$). At day 7, both OTR4120-treated and control wounds reached the CD34-staining peak, however, the OTR4120-treated wounds peaked to a significantly higher level, which was almost 20% higher than that of the control wounds (4.84 ± 0.08 vs. 4.12 ± 0.14, $p< 0.05$). At day 14, the score of CD34 staining of OTR4120-treated wounds was still higher than that of the control wounds (4.16 ± 0.24 vs. 3.25 ± 0.23, $p< 0.05$). Afterwards, the CD34-staining level declined, and at days 21 and 79 there were no significant differences of the CD34-staining score between the OTR4120-treated and the control wounds (3.44 ± 0.16 vs. 3.15 ± 0.12, at day 21; and 1.80 ± 0.08 vs. 1.80 ± 0.08, at day 79). It suggests that OTR4120 treatment boosts neovascularization at early stages of wound healing.

OTR4120 treatment reveals more VEGF and flt-1 proteins

To further elucidate the mechanism by which OTR4120 treatment stimulates neovascularization, the VEGF and VEGF-receptor 1 (flt-1) depositions and concentrations were also assayed. Both immunohistochemistry (Figure 3) and Western blot analysis (Figure 4) showed significant increases of VEGF and flt-1 proteins in OTR4120-treated wounds at days 3 and 7 after wounding. The VEGF deposition in OTR4120-treated wounds was scored twice as high as that in control wounds at day 3 (3.03 ± 0.48 vs.1.56 ± 0.26, $p< 0.05$), peaked at day 7 (3.48 ± 0.24 vs. 2.44 ± 0.30, $p< 0.05$), and declined thereafter to a level similar to that of control wounds at day 14 (2.12 ± 0.18 vs. 2.44 ± 0.28). Although the control wounds also had the VEGF-staining peak at day 7, it was 30% lower than that of OTR4120-treated wounds (2.44 ± 0.30 vs. 3.48 ± 0.24, $p< 0.05$). At day 14 and later time points, no difference was found between the treated and non-treated wounds. Similarly, at days 3 and 7, the depositions of flt-1 in OTR4120-treated wounds were all 1.3 times as high as that in control wounds (4.47 ± 0.18 vs. 3.56 ± 0.32, at day 3, $p< 0.05$; and 3.42 ± 0.19 vs. 2.73 ± 0.22, at day 7, $p< 0.05$). These immunohistochemical findings were supported by the results of Western blot analysis (Figure 4). Taken together, our data indicate that OTR4120 treatment stimulates angiogenesis at the early stages of wound healing.

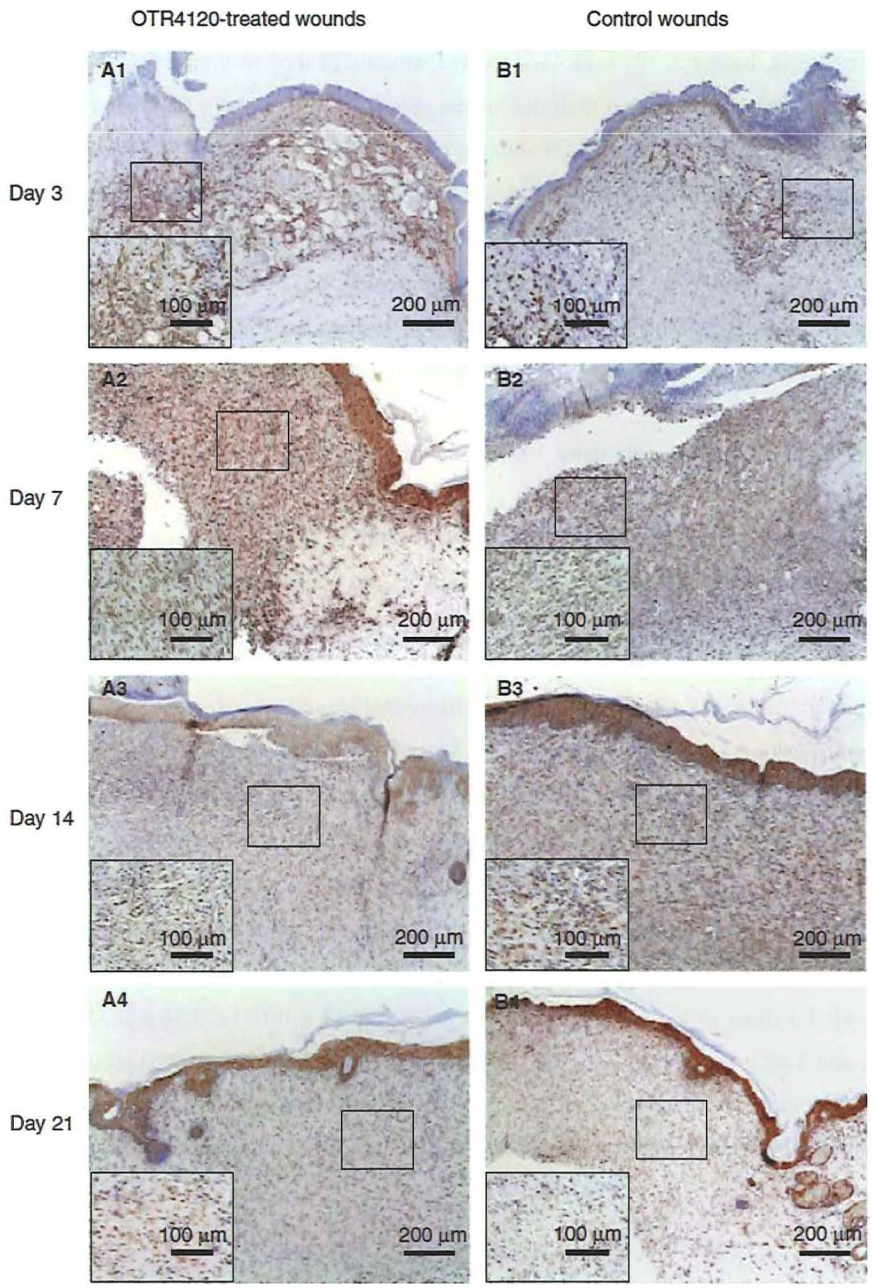

Figure 3. to be continued

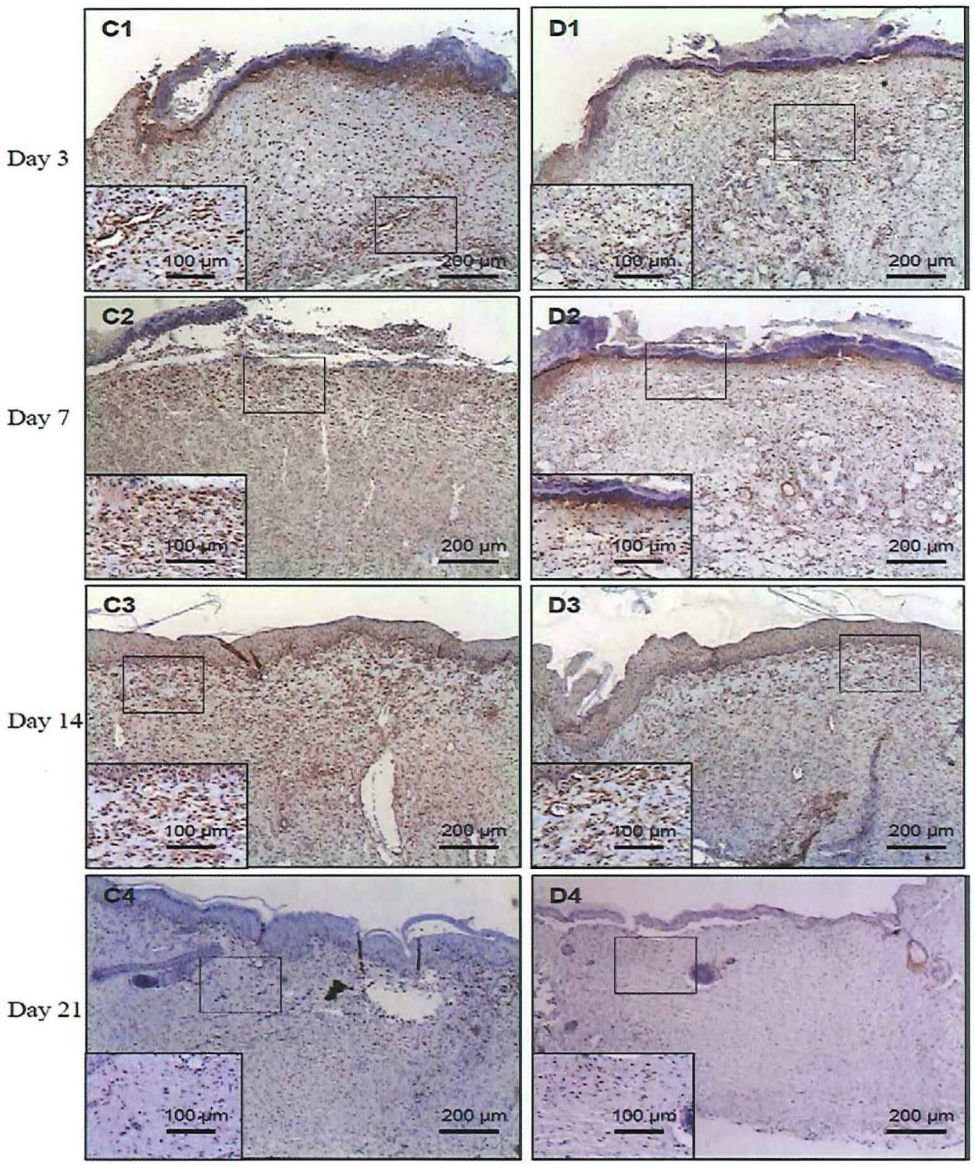

Figure 3. Effect of OTR4120 treatment on the depositions of vascular endothelial growth factor (VEGF) and VEGF-receptor 1 (flt-1) evaluated by immunohistochemistry. Representative VEGF-staining sections of OTR4120-treated wounds (A1-4) and control wounds (B1-4) at days 3, 7, 14, and 21 after wounding, respectively; Representative flt-1-staining sections of OTR4120-treated wounds (C1-4) and control wounds (D1-4) at days 3, 7, 14, and 21 after wounding, respectively. At days 3 and 7 after wounding, more increased VEGF and flt-1staining were found in the OTR4120-treated wounds than that in control wounds. Original magnification × 40. Original inset magnification × 200.

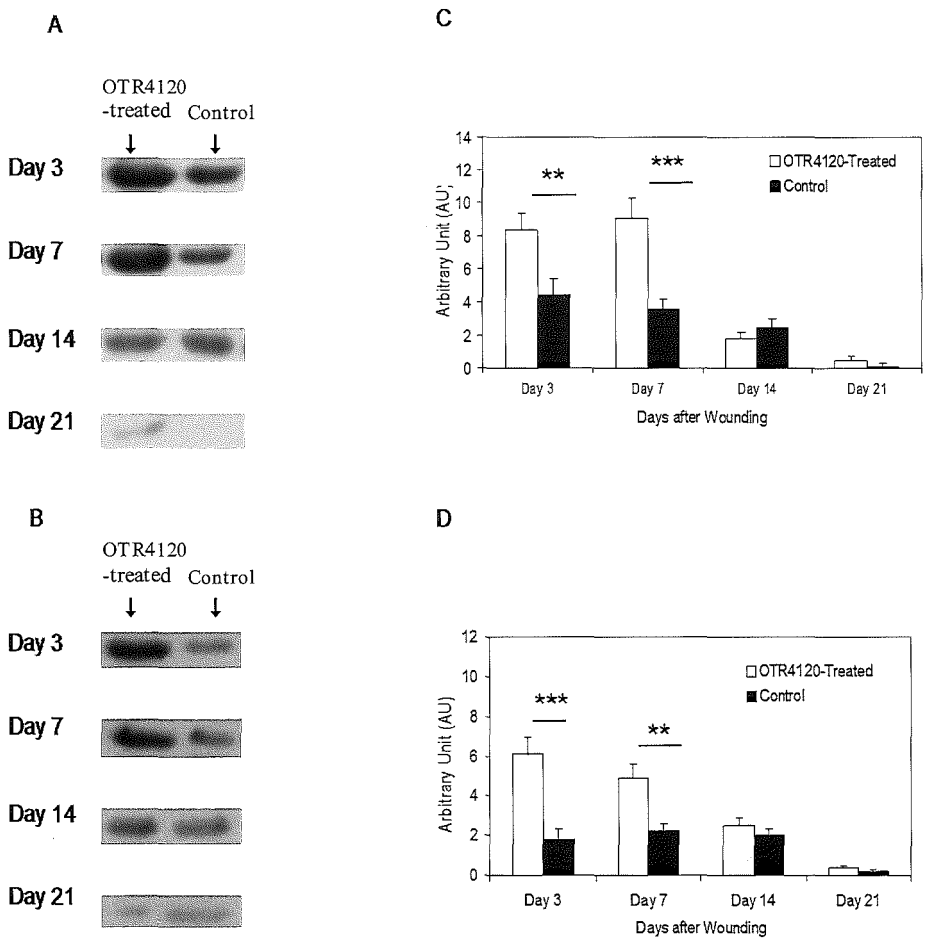

Figure 4. Effect of OTR4120 treatment on the vascular endothelial growth factor (VEGF) and VEGF-receptor 1 (flt-1) protein expressions assessed by Western blot analysis. Representative immunoblots of VEGF(A) and flt-1(B) at days 3,7,14, and 21 after wounding, respectively; Quantifications of the VEGF bands (C) and flt-1 bands (D). Data are presented as means ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences between OTR4120-treated wounds and control wounds.

OTR4120 treatment accelerates reepithelialization

The reepithelialization, expressed as the bilateral lengths of the inward migrating epithelial tongues, was measured at days 3, 7 and 14 after wounding. At day 14, the reepithelialization in both OTR4120-treated and control wounds was found fully accomplished (data not shown) making it impossible to establish the exact timing of the completion of reepithelialization. However, the neo-epidermis of the OTR4120-treated wounds was 80% longer at day 3, and 50% longer at day 7 than those of the control wounds (1.23 ± 0.13 vs. 0.65 ± 0.15 mm, at day 3, $p<$ 0.01; 2.94 ± 0.26 vs. 1.96 ± 0.20 mm, at day 7, $p<$ 0.01; Figure 5).

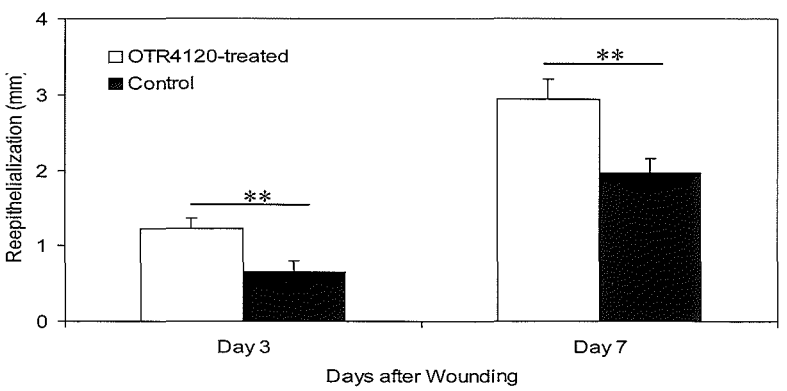

Figure 5. Effect of OTR4120 treatment on wound reepithelialization evaluated by H&E-stained histology at days 3 and 7 after wounding. Reepithelialization was assessed by measuring the bilateral lengths of the inward-migrating epithelial tongues from the wound margins to the tips of migrating epithelial tongues using a light microscope with an eyepiece micrometer. Data are presented as means ± SEM. $**p < 0.01$ indicates significant differences between OTR4120-treated wounds and control wounds.

OTR4120 treatment promotes epidermal proliferation

To further clarify the mechanism behind the accelerated reepithelialization observed in OTR4120-treated wounds, we evaluated the epidermal cell proliferation at day 7 after wounding using the cell proliferation marker BrdU immunohistochemistry. BrdU staining was found

mainly in the nuclei of the basal epidermal layer (data not shown). At the migrating epidermal tongue, the number of BrdU-positive epidermal cells in OTR4120-treated wounds was 76% higher than that in the control wounds (109 ± 9.0 vs. 62 ± 18, $p < 0.05$; Figure 6). No significant difference was found in epidermal cell proliferation in either total neo-epidermis or wound margin between OTR4120-treated and control wounds.

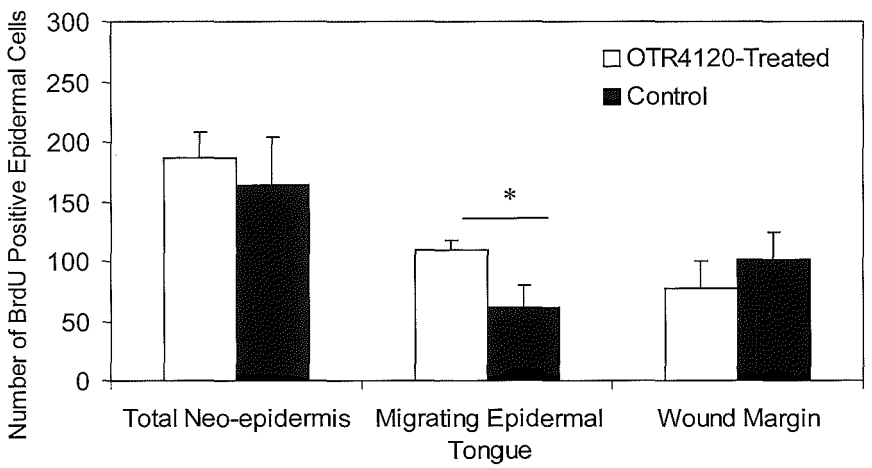

Figure 6. Effect of OTR4120 treatment on epidermal proliferation detected by the proliferation marker bromodeoxyuridine (BrdU) immunohistochemistry at day 7 after wounding. The number of BrdU-positive cells in the migrating epidermal tongue was increased compared to that in the total neo-epidermis and wound margin. Data are presented as means ± SEM. *$p < 0.05$ indicates the significant difference between the OTR4120-treated wounds and the control wounds.

OTR4120 treatment increases granulation tissue deposition (neo-dermis reconstruction)

The formation of granulation tissue or neo-dermis was measured on H&E-staining sections at all experimental time points. OTR4120 treatment significantly increased the granulation tissue deposition or neo-dermis formation at all intervals except for day 14 (1.22 ± 0.07 vs. 0.94 ± 0.05 mm, at day 3, $p < 0{,}01$; 1.46 ± 0.10 vs. 1.06 ± 0.12 mm, at day 7, $p < 0.05$; 1.04 ± 0.09 vs. 0.74 ± 0.07 mm, at day 21, $p < 0{,}05$; and 1.06 ± 0.14 vs. 0.73 ± 0.04 mm, at day 79, $p < 0{,}05$; Figure 7).

At day 79 after wounding, the thickness of the neo-dermis in OTR4120-treated wounds was 60% that of the surrounding unwounded normal dermis, whereas neo-dermis thickness in control wounds reached only 40% that of the normal dermis.

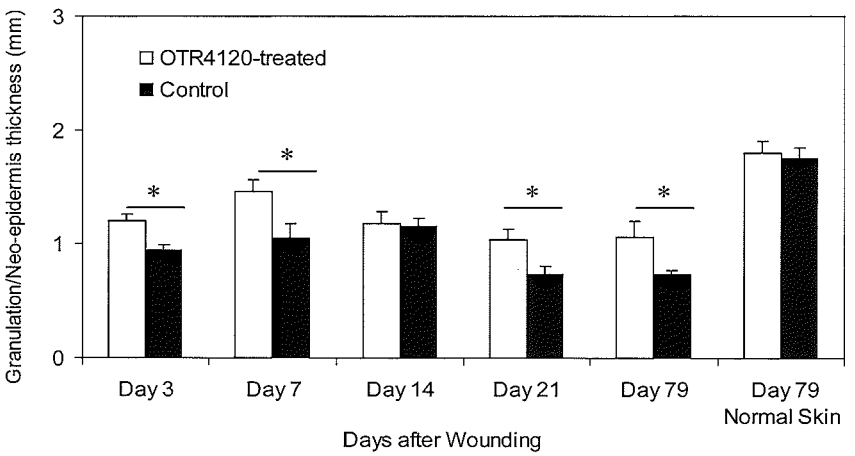

Figure 7. Effect of OTR4120 treatment on formation of granulation tissue or neo-dermis assessed by the H&E-stained histology at all experimental time points. Data are presented as means ± SEM. *$p < 0.05$, and **$p < 0.01$ indicate significant differences between the OTR4120-treated wounds and the control wounds.

OTR4120 treatment promotes collagen maturation

Collagen maturation was assessed by examining the collagen solubility profile in OTR4120-treated and control wounds. This was achieved by measuring the collagen contents in salt-soluble collagen (SSC), acid-soluble collagen (ASC), pepsin-soluble collagen (PSC), and insoluble collagen (ISC) fractions. At day 14 after wounding, the solubility profile in the OTR4120-treated wounds revealed a 41% increase in SSC and a 37% increase ISC, and a 13% decrease in PSC contents compared with their control wounds (34.25 ± 2.21 vs. 24.37 ± 2.08 μg/mg, SSC, $p< 0.05$; 36.98 ± 2.27 vs. 26.97 ± 2.27 μg/mg, ISC, $p< 0.01$; and 112.98 ± 5.34 vs.

129.85 ± 5.96 μg/mg, PSC, $p < 0.05$; Figure 8). No statistically significant difference was observed in the ASC content between OTR4120-treated and control wounds.

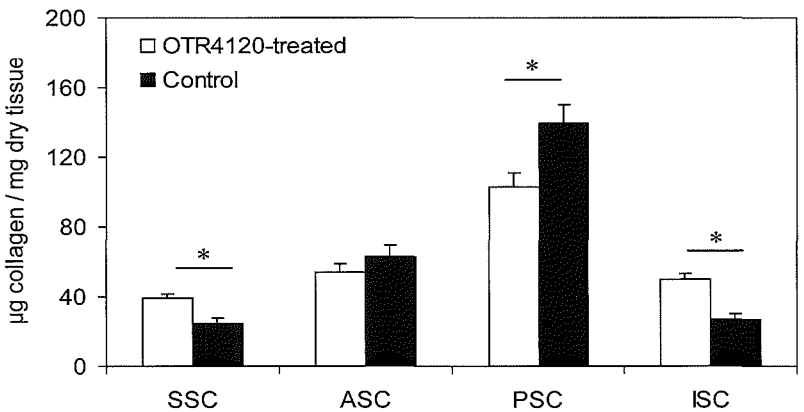

Figure 8. Effect of OTR4120 treatment on collagen maturation evaluated by examining the collagen solubility profile in wound tissue at day 14 after wounding. Collagen solubility profile was achieved by successive extractions of collagen in neutral salt buffer, acetate buffer, and acetate-pepsin buffer. The collagen contents in the obtained salt-soluble collagen (SSC), acid-soluble collagen (ASC), pepsin-soluble collagen (PSC), and insoluble collagen (ISC) were assessed by Sircol Collagen Assay kit. Data are presented as means ± SEM. $*p < 0.05$ and $**p < 0.01$ indicate significant differences between OTR4120-treated wounds and control wounds.

DISCUSSION

An important concept in normal wound healing is that of feedback/feedforward loops. During the coordinated acute wound healing process, each stage proceeds depending on a feedforward from the previous stage. When a progressive stage reaches a critical point, at which it is no longer dependent on the feedforward from the previous stage to continue, it will send a feedback to inhibit or turn off the previous stage. Therefore, each progressive stage is not only capable of initiating the next stage, but also of self-limiting [13]. These interlocking feedback/feedforward loops allow an orderly and coordinated progression of wound healing and prevent any one stage from inhibiting and/or perpetuating. In the regulation of these feedback/feedforward loops, the

ECM plays a crucial role in controlling tissue architecture and regulating cell behaviours. As an important family of the ECM components, HS-GAGs are significantly involved in storing, protecting, and sequestrating various proteins and cytokines that are implicated in modulation of cell adhesion, migration, proliferation, and differentiation in wound healing [3]. However, after tissue injury, HS-GAGs and other components of the ECM can be constantly degraded by the prolonged inflammation. The degraded ECM will not protect any longer the matrix proteins and growth factors. Consequently, the interlocking feedback/feedforward loops will be disconnected and the acute wounds will become impaired [12-13].

OTR4120 is a mammalian enzyme resistant HS-GAG mimetic that is synthesized to replace and take over the role of the natural HS-GAGs which are degraded during wound healing, therefore, OTR4120 is predicted to protect and recruit the ECM proteins as well as cell communications back to the wounded ECM so cells will see a wound microenvironment close to that in normal tissue. In this study we demonstrate that OTR4120 treatment improves the quality of cutaneous wound healing through stimulating inflammation resolution, boosting angiogenesis and reepithelialization, increasing the granulation tissue formation, and accelerating collagen synthesis and maturation. This makes OTR4120 a potent matrix homeostasis optimizer that improves the quality of cutaneous wound healing and is, therefore, a promising aid for regenerative medicine in clinical practice.

Inflammation in an acute wound occurs to remove necrotic tissue, debris and bacterial contaminates as well as to recruit and activate fibroblasts. However, healing proceeds only after inflammation is controlled. Wounds that fail to heal are usually entrapped in a self-sustaining cycle of chronic inflammation and therefore the wounds are not matured enough to move forward to the next stage of wound healing [13, 37]. The home for inflammation to return is resolution. Although the exact time of resolution in this study is not located in our experimental time points, the finding that inflammation assessed by the number of macrophages was reduced by a third in OTR4120-treated wounds from days 7 to 21 after wounding allows us to predict that inflammation resolution in OTR4120-treated wounds was stimulated. Sending inflammatory cells home early contributes to the better quality of acute wound healing after OTR4120 application. Although the exact molecular mechanism of this anti-inflammatory effect of OTR4120 treatment remains unclear, a recent study demonstrated that VEGF induced

macrophage apoptosis through stimulation of an tumour necrosis factor [38]. This result tempts us to predict that the rapid resolution of inflammation following OTR4120 treatment may be due to an increased VEGF synthesis and/or the consequence of the protection of VEGF by OTR4120 application, because an increased deposition and content of VEGF were also found after OTR4120 treatment in this study.

In addition to stimulating inflammation resolution, OTR4120 treatment increases neovascularization at the early stages of wound healing. In the current study, the immunostaining score of newly formed blood vessels at day 3 in OTR4120-treated wounds was 80% higher than that in control wounds. This effect of OTR4120 treatment, in stimulating neovascularization, may be related to the current finding that OTR4120 treatment leads to increased contents of VEGF and flt-1 (VEGF receptor-1) also at day 3 after wounding. This finding suggests that OTR4120 treatment is effective in synthesizing more VEGF, and/or in protecting VEGF from proteolysis and potentiating VEGF bioavailability. The latter has been documented in a previous study, where OTR4120 was shown high-affinity binding to VEGF as compared with heparin, and also OTR4120 potentiated the affinity of VEGF for its receptors [25]. Therefore, the angiogenic potential of OTR4120 treatment may be the result of the capability of OTR4120 to protect and sequester endogenous growth factors (such as VEGF) in the matrix which might preserve the natural signalling of angiogenesis between cell-cell and cell-matrix interactions, thereby stimulating neovascularization and improving the quality of tissue repair.

Reepithelialization is one of the basic mechanisms of cutaneous wound healing. It not only prevents infection and excessive water loss but also improves functional outcome by minimizing scarring [39]. It was reported that human hypertrophic burn scars were more likely to occur if wound closure required more than 3 weeks after burn injury [40]. In this full-thickness excisional wound model, reepithelialization is fully investigated, because the excisional wound creates an open defect, where reepithelialization is accomplished by the migration of epidermal cells from the surrounding wound margins towards their center, forming the migrating epidermal tongues [41]. The present data show that, at day 3 after wounding, reepithelialization in OTR4120-treated wounds is almost twice as fast as that in the control wounds. A beneficial

effect of OTR4120 treatment on accelerating reepithelialization was also shown in a murine necrotic skin ulcer model [27].

The present study finds two potential means whereby OTR4120 treatment may help accelerate reepithelialization. We observed that epidermal keratinocyte proliferation was significantly increased near the migrating epidermal tongues, but not at the wound margins. This finding is consistent with the two complementary mechanisms of reepithelialization: (1) near the wound margin, keratinocytes stratify to regenerate a differentiated epidermis endowed with barrier properties, and (2) at the tip of the migrating neo-epidermis, keratinocytes crawl over each other. These combined mechanisms will create a highly proliferative index near the migrating epidermal tongues than at the wound margins, where the differentiation and regeneration of the neo-epidermis dominates [41]. The present study confirms the existence of such mechanisms. Another potential reason, which is related to the effect of OTR4120 treatment in accelerating wound reepithelialization, is the increased contents of VEGF and flt-1 after OTR4120 treatment observed in this study. It was reported that the biological activity of VEGF during wound healing not only increases the growth and permeability of vasculature but also induces the proliferation of non-endothelial cell types, such as keratinocytes [35, 42]. VEGF was shown to promote proliferation in human epidermal keratinocytes, which was flt-1 dependent. flt-1 was also shown to be intensely stained in the murine epidermal keratinocytes during full-thickness dorsal wound repair [35]. Overall, those findings of OTR4120 treatment, stimulating angiogenesis and reepithelialization, while resolving inflammation, may be of particular interest when considering an anti-inflammatory and/or angiogenesis-promoting therapy to improve the quality of chronic wounds.

We demonstrated previously that OTR4120 treatment increased the cutaneous wound breaking strength [15]. It has been known that the strength of scar tissue is much less than that of normal skin [43]. The better restoration of wound skin strength contributes to the better restitution of biomechanical functioning of dermal tissue. The development of wound strength is closely related to the collagen remodeling and maturation, especially the cross-linked collagen synthesis [44]. In the present study, we demonstrate that OTR4120 treatment enhances the granulation tissue formation and leads to a 45% increased thickness of neodermis compared with control wounds at day 79 after wounding. The collagen solubility assay further explains the beneficial

effects of OTR4120 treatment on matrix reconstruction. The salt-soluble collagen (SSC) represents the tropocollagen monomers that are newly synthesized within the last few hours before harvest, thereafter, this SSC will crystallise into collagen fibrils and become salt insoluble [45]. The acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) fractions can be considered degradation intermediates of insoluble collagen (ISC). ISC is covalently cross-linked collagen that contributes to tissue strength. In this study, both SSC and ISC percentages in OTR4120-treated wounds were 4% increased compared with control wounds, suggesting a rapid collagen synthesis and an increased maturation in wound tissue after OTR4120 treatment. The increased amount of ISC, which contributes to the cross-linked collagen synthesis, especially supports our previous biomechanical findings after OTR4120 application [28]. The decreased amount of PSC in OTR4120-treated wounds (7% less than that in controls) also indicates that OTR4120 treatment increases the resistance of wound tissue to pepsin digestion. Overall, OTR4120 treatment leads to a reduction in the ratio of soluble collagen to insoluble collagen [(SSC+ASC+ PSC): ISC] from 6:1 in OTR4120-treated wounds to 8:1 in control wounds. This observed collagen solubility profile suggests an increased collagen synthesis and maturation after TR4120 treatment. Considering this together with our previous study OTR4120-treated wounds possess enhanced tensile strength accompanied with an increased content of insoluble cross-linked collagen, suggesting that OTR4120 treatment leads to a superior wound healing.

In summary, we demonstrate that OTR4120 treatment improves cutaneous wound repair in rat full-thickness excisional wounds. Its mechanism consists, at least in part, of resolving inflammation, stimulating neovascularization, and increasing collagen synthesis and maturation. Our findings suggest that OTR4120 has a therapeutic potential in treating impaired wounds characterized by deficient angiogenesis and prolonged inflammation.

ACKNOWLEDGEMENTS

This research was supported by grants from the Nuts Ohra Foundation (the Netherlands), grant No. SNO-T-0-0501-159.

REFERENCES

1. Martin, P., Wound healing--aiming for perfect skin regeneration. Science, 1997. 276(5309): p. 75-81.
2. Thomas, D.W., et al., Cutaneous wound healing : A current perspective. Journal of Oral and Maxillofacial Surgery, 1995. 53(4): p. 442-447.
3. Bosman, F.T. and I. Stamenkovic, Functional structure and composition of the extracellular matrix. J Pathol, 2003. 200(4): p. 423-8.
4. Sommer, A. and D.B. Rifkin, Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. J Cell Physiol, 1989. 138(1): p. 215-20.
5. Lyon, M. and J.T. Gallagher, Bio-specific sequences and domains in heparan sulphate and the regulation of cell growth and adhesion. Matrix Biol, 1998. 17(7): p. 485-93.
6. Rahmoune, H., et al., Interaction of heparan sulfate from mammary cells with acidic fibroblast growth factor (FGF) and basic FGF. Regulation of the activity of basic FGF by high and low affinity binding sites in heparan sulfate. J Biol Chem, 1998. 273(13): p. 7303-10.
7. Allen, B.L., M.S. Filla, and A.C. Rapraeger, Role of heparan sulfate as a tissue-specific regulator of FGF-4 and FGF receptor recognition. J Cell Biol, 2001. 155(5): p. 845-58.
8. Park, P.W., O. Reizes, and M. Bernfield, Cell surface heparan sulfate proteoglycans: selective regulators of ligand-receptor encounters. J Biol Chem, 2000. 275(39): p. 29923-6.
9. Hacker, U., K. Nybakken, and N. Perrimon, Heparan sulphate proteoglycans: the sweet side of development. Nat Rev Mol Cell Biol, 2005. 6(7): p. 530-41.
10. Nwomeh, B.C., et al., Dynamics of the matrix metalloproteinases MMP-1 and MMP-8 in acute open human dermal wounds. Wound Repair Regen, 1998. 6(2): p. 127-34.
11. Barrick, B., E.J. Campbell, and C.A. Owen, Leukocyte proteinases in wound healing: roles in physiologic and pathologic processes. Wound Repair Regen, 1999. 7(6): p. 410-22.
12. Eming, S.A., T. Krieg, and J.M. Davidson, Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol, 2007. 127(3): p. 514-25.
13. Menke, N.B., et al., Impaired wound healing. Clin Dermatol, 2007. 25(1): p. 19-25.
14. Grinnell, F. and M. Zhu, Fibronectin degradation in chronic wounds depends on the relative levels of elastase, alpha1-proteinase inhibitor, and alpha2-macroglobulin. J Invest Dermatol, 1996. 106(2): p. 335-41.
15. Grinnell, F., C.H. Ho, and A. Wysocki, Degradation of fibronectin and vitronectin in chronic wound fluid: analysis by cell blotting, immunoblotting, and cell adhesion assays. J Invest Dermatol, 1992. 98(4): p. 410-6.

16. Campbell, E.J., Preventive therapy of emphysema. Lessons from the elastase model. Am Rev Respir Dis, 1986. 134(3): p. 435-7.
17. Wysocki, A.B., et al., Temporal expression of urokinase plasminogen activator, plasminogen activator inhibitor and gelatinase-B in chronic wound fluid switches from a chronic to acute wound profile with progression to healing. Wound Repair Regen, 1999. 7(3): p. 154-65.
18. Norgauer, J., et al., Elevated expression of extracellular matrix metalloproteinase inducer (CD147) and membrane-type matrix metalloproteinases in venous leg ulcers. Br J Dermatol, 2002. 147(6): p. 1180-6.
19. Saarialho-Kere, U.K., Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers. Arch Dermatol Res, 1998. 290 Suppl: p. S47-54.
20. Wysocki, A.B., L. Staiano-Coico, and F. Grinnell, Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. J Invest Dermatol, 1993. 101(1): p. 64-8.
21. Wlaschek, M., et al., Protease inhibitors protect growth factor activity in chronic wounds. Br J Dermatol, 1997. 137(4): p. 646.
22. Lauer, G., et al., Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. J Invest Dermatol, 2000. 115(1): p. 12-8.
23. Lauer, G., et al., Generation of a novel proteolysis resistant vascular endothelial growth factor165 variant by a site-directed mutation at the plasmin sensitive cleavage site. FEBS Lett, 2002. 531(2): p. 309-13.
24. Garcia-Filipe, S., et al., RGTA OTR4120, a heparan sulfate mimetic, is a possible long-term active agent to heal burned skin. J Biomed Mater Res A, 2007. 80(1): p. 75-84.
25. Rouet, V., et al., A synthetic glycosaminoglycan mimetic binds vascular endothelial growth factor and modulates angiogenesis. J Biol Chem, 2005. 280(38): p. 32792-800.
26. Zuijdendorp, H.M., et al., Significant reduction in neural adhesions after administration of the regenerating agent OTR4120, a synthetic glycosaminoglycan mimetic, after peripheral nerve injury in rats. J Neurosurg, 2008. 109(5): p. 967-73.
27. Barbier-Chassefiere, V., et al., Matrix therapy in regenerative medicine, a new approach to chronic wound healing. J Biomed Mater Res A, 2008.
28. Tong, M., et al., RGTA OTR 4120, a heparan sulfate proteoglycan mimetic, increases wound breaking strength and vasodilatory capability in healing rat full-thickness excisional wounds. Wound Repair Regen, 2008. 16(2): p. 294-9.
29. Chebbi, C.K., et al., [Pilot study of a new matrix therapy agent (RGTA OTR4120) in treatment-resistant corneal ulcers and corneal dystrophy]. J Fr Ophtalmol, 2008. 31(5): p. 465-71.
30. Barritault, D. and J.P. Caruelle, [Regenerating agents (RGTAs): a new therapeutic approach]. Ann Pharm Fr, 2006. 64(2): p. 135-44.

31. Rouet, V., et al., Heparin-like synthetic polymers, named RGTAs, mimic biological effects of heparin in vitro. J Biomed Mater Res A, 2006. 78(4): p. 792-7.
32. Holness, C.L. and D.L. Simmons, Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood, 1993. 81(6): p. 1607-13.
33. Fina, L., et al., Expression of the CD34 gene in vascular endothelial cells. Blood, 1990. 75(12): p. 2417-26.
34. Nissen, N.N., et al., Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. Am J Pathol, 1998. 152(6): p. 1445-52.
35. Wilgus, T.A., et al., Novel function for vascular endothelial growth factor receptor-1 on epidermal keratinocytes. Am J Pathol, 2005. 167(5): p. 1257-66.
36. deFazio, A., et al., Immunohistochemical detection of proliferating cells in vivo. J Histochem Cytochem, 1987. 35(5): p. 571-7.
37. Martin, P. and S.J. Leibovich, Inflammatory cells during wound repair: the good, the bad and the ugly. Trends in Cell Biology, 2005. 15(11): p. 599-607.
38. Melissa L. Petreaca, M.Y., Carl Ware, Manuela M. Martins-Green, Vascular endothelial growth factor promotes macrophage apoptosis through stimulation of tumor necrosis factor superfamily member 14 (TNFSF14/LIGHT). Wound Repair and Regeneration, 2008. 16(5): p. 602-614.
39. Braiman-Wiksman, L., et al., Novel insights into wound healing sequence of events. Toxicol Pathol, 2007. 35(6): p. 767-79.
40. Deitch, E.A., et al., Hypertrophic burn scars: analysis of variables. J Trauma, 1983. 23(10): p. 895-8.
41. Laplante, A.F., et al., Mechanisms of wound reepithelialization: hints from a tissue-engineered reconstructed skin to long-standing questions. Faseb J, 2001. 15(13): p. 2377-89.
42. Frank, S., et al., Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem, 1995. 270(21): p. 12607-13.
43. Levenson, S.M., et al., The Healing of Rat Skin Wounds. Ann Surg, 1965. 161: p. 293-308.
44. Muehlberger, T., et al., The effect of topical tretinoin on tissue strength and skin components in a murine incisional wound model. J Am Acad Dermatol, 2005. 52(4): p. 583-8.
45. Jackson, D.S. and J.P. Bentley, On the significance of the extractable collagens. J Biophys Biochem Cytol, 1960. 7: p. 37-42.

CHAPTER 4

Heparan sulfate glycosaminoglycan mimetic improves pressure ulcer healing in a rat model of cutaneous ischemia-reperfusion injury

Miao Tong, Bastiaan Tuk, Ineke M. Hekking, Mieke M. Pleumeekers, Mireille B. Boldewijn, Steven E.R. Hovius, Johan W. van Neck

Wound Repair Regen 2011; 19: 505-514

ABSTRACT

Pressure ulcers are a major clinical problem with a large burden on healthcare resources. This study evaluated the effects of the heparan sulfate glycosaminoglycan mimetic, OTR4120, on pressure ulceration and healing. Ischemia-reperfusion was evoked to induce pressure ulcers by external clamping and then removal of a pair of magnet disks on rat dorsal skin for a single ischemic period of 16 hours. Immediately after magnet removal, rats received an intramuscular injection of OTR4120 weekly for up to 1 month. During the ischemic period normal skin perfusion was reduced by at least 60%, and at least 20-45% reperfused into the ischemic region after compression release. This model caused sustained skin incomplete necrosis for up to 14 days and led to grade 2-3 ulcers. OTR4120 treatment decreased the area of skin incomplete necrosis and degree of ulceration. OTR4120 treatment also reduced inflammation and increased angiogenesis. In OTR4120-treated ulcers, the contents of vascular endothelial growth factor, platelet-derived growth factor, and transforming growth factor beta-1 were increased. Moreover, OTR4120 treatment promoted early expression of alpha-smooth muscle actin and increased collagen biosynthesis. Long-term restoration of wounded tissue biomechanical strength was significantly enhanced after OTR4120 treatment. Taken together, we conclude that OTR4120 treatment reduces pressure ulcer formation and potentiates the internal healing bioavailability.

INTRODUCTION

Pressure ulcers are a significant healthcare concern, particularly for the physically limited or bedridden elderly [1]. The treatment of pressure ulcers is a difficult medical problem that requires a significant amount of healthcare resources [2-3]. Moreover, this load will undoubtedly increase with the drastic increase in the elderly population [3].

Conventional treatments of pressure ulcers have mainly relied on passive interventions, such as pressure-relieving devices, dressings, and antimicrobial agents [4-5]. All these therapies mainly focus on the management of underlying causes and/or the external interventions in only a certain aspect of the wound-healing process. However, as with most complex conditions, chronic wounds show a wide variability and heterogeneity in healing process itself, among patients, and also in the complexities associated with the pathophysiology of the disease that can impair the healing process [6-7]. Therefore, an ideal treatment of pressure ulcers should be healing-phase dependent and also take into account the individual patients' characteristics. This personalized treatment of chronic wounds is a considerable challenge for the clinicians.

One aspect of a chronic wound healing mechanism that should not be neglected is the tissue self-regenerating capability. The intrinsic regenerating capability would be stimulated and maximized only when the healing environment is regulated and in homeostasis. Extra-cellular matrix (ECM) is largely involved in healing environment homeostasis. Heparan sulfate glycosaminoglycans (HS-GAGs) play an important role in maintaining matrix homeostasis. HS-GAGs are not only structural elements of tissue architecture, but are also known as regulators of the bioavailability of a large variety of locally synthesized HS-bound polypeptides. These include chemokines, morphogens, and growth factors (GFs), such as fibroblast growth factor family (FGFs), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [8]. However, in chronic wounds, the highly proteolytic microenvironment can disrupt the ECM including the HS-GAGs [9-10]. Through this degradation, the orchestrating role of HS-GAG in GF sequestration for wound healing is lost and the tissue healing homeostasis can be disturbed. The disturbed homeostasis is a cardinal feature of chronic wounds and also a major obstacle, preventing the effectiveness of many treatments [11-14].

Our strategy of wound treatments is to potentiate the tissue intrinsic self-regeneration capability by regulating and facilitating the wound bio-microenvironment using synthetic matrix modulators. OTR4120, a HS-GAG mimetic, is designed to resistant to all known mammalian enzymes, including proteases and glycanases, and bacterial degradation enzymes. It can replace the degraded HS-GAGs and bind to the free heparin-binding sites that become available following HS-GAGs degradation on the matrix proteins. OTR4120 thereby provides protection and sequestration sites for both newly formed GFs and free GFs released following HS-GAG degradation during wound healing. This allows the cells to face a renewed microenvironment, closely related to the ECM in the uninjured state, thereby restoring homeostasis.

We previously reported that OTR4120 treatment improves surgically full-thickness excisiontal wound healing in rats [15-16]. In the present study, the application of OTR4120 was extended to an ischemia-reperfusion (I-R) injury-mediated pressure ulcer healing. The effects of OTR4120 treatment in ulceration and ulcer healing were investigated by evaluating skin incomplete necrosis, inflammation, angiogenesis, collagen biosynthesis, and wound biomechanical strength.

MATERIALS AND METHODS

Animals and groups

WAG/RijHsd female rats (136, 10-11 weeks old; 150-200 grams; purchased from Harlan, Zeist, the Netherlands) were used. Rats were randomly allocated to nine groups to serve nine experimental time points (i.e. day 1, n=8; day 2, n=8; day 3, n=16; day 4, n=8; day 7, n=16; day 14, n=24; day 21, n=24; day 42, n=24, and day 84, n=8). In each group, the rats were equally randomized into an OTR4120-treated group and a physiological saline-treated (control) group. Rats were exposed to a 12 h light/dark cycle and fed a standard laboratory diet (Hope Farms, Woerden, the Netherlands) with food and water available ad libitum. Prior to the procedure, rats were allowed to acclimatize to their environment for 1 week.

The experimental protocol was approved by the local Animal Experiments Committee and adhered to the rules prescribed in the national Animals Act, which implements the 'Guidelines

on the protection of experimental animals' by the Council of Europe, 1986: Directive 86/609/EC.

Model creation: magnet compression procedure

The magnet compression model that has been reported previously was used with some modifications [17]. Briefly, rats were anesthetized using isoflurane (Rhodia Organique Fine Ltd., Bristol, UK) in oxygen as carrier. The dorsal hair was shaved and the area was cleansed with 70% alcohol. The location for the magnet clamping, vertically 4 cm caudal to the occipital bone of the cranium, and 3 cm apart from the centers of the two magnets, was marked; full-thickness skin was then pulled up and placed between a pair of magnet disks (PIC GmbH, Wendelstein, Germany) that had a 15 mm diameter and were 7 mm thickness, with an average weight of 3 g and 2,300 G magnetic forces. Immediately after the magnets were applied, animals were left to emerge from anaesthesia and individually housed in non-magnetic cages. After a constant 16 hours clamping, magnets were released. The compressed area was left uncovered.

OTR4120 treatment

OTR4120 is an alpha1-6 carboxymethyl sulphated glucose polymer of average molecular mass of 77kD [18]. Lyophilized OTR4120 was rehydrated in a physiological salt solution (0.9 % NaCl, Braun Melsungen AG, Germany) at a concentration of 13 μM OTR4120 (1mg/mL) for intramuscular (i.m.) injection.

Immediately after magnet removal, rats were randomly assigned to receive an i.m. injection of OTR4120 on the right thigh at a dose of 13 nmol/kg bodyweight (1 mg/ kg bodyweight, in a total volume of 150 to 200 μL according to the bodyweight) or a same volume of physiological saline weekly for up to 1 month.

Model evaluation: skin perfusion and reperfusion, incomplete necrosis, ulcer grade, and ulcer closure percentage

The dynamic change in skin perfusion at the compressed area after magnet removal was monitored using a Laser Doppler Perfusion Monitor (PeriFlux System 5000, Perimed AB, Stockholm, Sweden). The area to be compressed was marked and its perfusion was measured before magnets were applied. The pre-measured skin perfusion in the area to be compressed was designated as 'normal' skin perfusion.

For analysis of skin necrosis, ulcers were photographed with a digital camera (Nikon Coolpix 4,500, Nikon Ltd., Tokyo, Japan) using a graduated ruler as reference for image calibration. Skin incomplete necrosis was defined by bluish-purple discoloration/cyanosis. Using the Image J program, the border of the cyanosis area was manually traced and the area within the outlined border was computerized. The percentage of skin incomplete necrosis was calculated for data analysis according to the following formula:

% Incomplete necrosis area = [(Area of incomplete necrosis / Compressed area (1.77 cm^2)]× 100%

When cyanosis (incomplete necrosis) progressed to complete necrosis (scab) or an open ulcer, it was regarded as 100% incomplete necrosis.

Ulcer stage was also evaluated according to the grading system of the National Pressure Ulcer Advisory Panel (NPUAP) [2-3]. In this system, pressure ulcers are classified as superficial pressure ulcers with intact skin (Grade 1); partial-thickness of skin loss involving epidermis, dermis, or both (Grade 2); full-thickness skin loss involving damage to subcutaneous tissue (Grade 3); and full-thickness skin loss with extensive destruction of underlying tissue (Grade 4). In this study, the superficial pressure ulcers with more than 25% incomplete necrosis were classified as Grade 1.5.

The closed ulcers' percentage at day 21 was counted in both OTR4120-treated and control groups.

Sampling and preparation

Rats were euthanized at each time point. In rats assigned for breaking strength measurement, the dorsal pelts were excised. For all other rats, the ulcer tissues, including 2 mm of the surrounding normal skin, were excised and cut into halves. One half was fixed in 10% phosphate-buffered formalin for histology and immunohistochemistry (IHC). For the other half, the surrounding normal skin tissue was excised and only the wounded tissue was snap-frozen in liquid nitrogen, and homogenized using Mikro-Dismembrator (B. Braun Biotech International, Melsungen, Germany). The homogenates were kept at -80°C for Western blot analysis and hydroxyproline content measurement.

Breaking strength measurement

Breaking strength was measured as described previously [15]. Briefly, the excised dorsal pelt was cut into standardized dumbbell-shaped skin strips, each centred by a segment of the wound. The strip was fixed perpendicularly between the two clips of a tensiometer and subjected to a constant strain rate of 60mm/minute using a 1.0kg force transducer. Breaking strength was recorded as the maximum load (Newton) measured before skin failure. The ratio of ulcer breaking strength to that of local normal skin breaking strength was calculated for data analysis.

Immunohistochemistry

To evaluate inflammation and angiogenesis, CD68 (a monocyte/macrophage marker, purchased from AbD Serotec, Düsseldorf, Germany) and CD34 (an endothelial cell marker, purchased from R&D Systems, Minneapolis, MN) were used, respectively, and detected at ulcer tissues biopsied at all nine time points using avidin-biotin-peroxidase complex immunohistochemistry as described previously [16].

For evaluation of staining, the entire area of the wound on the slide was observed under a microscopy using different magnifications, and the overview of the positive-signal density was scored semi-quantitatively as 1 (absent), 2 (low), 3 (medium), 4 (strong), and 5 (very strong).

To minimize the subjectivity between observers, four observers, who were blinded to the treatments, carried out the scoring independently. The median of their scores was calculated for each individual sample.

Western blot analysis

The expressions of VEGF, VEGF receptor-1 (fit-1), PDGF-A (Santa Cruz Biotechnology, Santa Cruz, CA), TGF-β_1 (R&D Systems), and α-SMA (Abcam, Cambridge, MA) were detected in ulcer tissues on days 3, 7, 14 and 42, respectively, by Western blot analysis as described previously [16].

Hydroxyproline content measurement

Hydroxyproline content was measured using colorimetric assay [19]. Briefly, homogenates of wound tissue were hydrolyzed in 6 N HCl in a ratio of 10 mg tissue to 1 mL 6 N HCl at 100°C for 24 hours. The remaining HCl was neutralized with 2.5 N NaOH and 10-fold diluted with deionized H_2O. Then 250 µL of 0.05 M chloramine T (Sigma-Aldrich) solution was added to 250 µL of the neutralized/diluted solution and incubated for 20 minutes at room temperature. The solution was mixed with 250 µl of 3.15 M perchloric acid and left to stand for 5 minutes at room temperature. Then 250 µL of 20% p-dimethylaminobenzaldehyde (Sigma-Aldrich) was added and the resulting mixture was incubated for 20 minutes at 60°C. Absorbance at 557 nm was measured using a VersaMax microplate reader (Molecular Devices, CA). The hydroxyproline content was determined according to a standard curve, and normalized to a milligram of wound tissue homogenate for data analysis.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Statistical calculations were performed using SPSS software, version 11 (Chicago, IL). The Mann-Whitney U-test was

carried out to compare results between groups. A P value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

The magnet compression model conducted skin ischemia and reperfusion

Skin ischemia and reperfusion was verified by monitoring changes in skin perfusion in response to the release of compression. In the first minute after magnet removal, skin perfusion was 40% that of normal skin perfusion. Within 30 minutes, skin perfusion had increased to the maximum of 85% (reactive hyperaemia) and then dropped to a plateau value of 60% (Figure 1). In this model, it is not possible to monitor the exact amount of skin perfusion when skin is under pressure. However, it is feasible that the decreased perfusion in compressed skin is lower than the perfusion measured during the first minute after compression release, due to the initial reactive hyperaemia. Therefore, the decrease in skin perfusion during compression is at least 60% of normal skin perfusion, and the reperfusion after compression release is then at least 20-45% of normal skin perfusion.

The magnet compression model induced sustained incomplete necrosis that was suppressed by OTR4120 treatment

The mean of the percentage of the area of incomplete necrosis area was 24 ± 3% on day 1, 31 ± 3% on day 2, 36 ± 1% on day 3, and 41 ± 2% on day 7. No significant different was found between OTR4120-treated animals and controls within the first week after compression removal. On day 14, the mean percentage of incomplete necrosis reached the maximum of 90 ± 3%, which was significantly reduced in the OTR4120-treated ulcers compared with controls (82 ± 6% vs. 98 ± 2%, $p< 0.01$).

The magnet compression model created grade 2-3 ulcers and the ulceration was attenuated by OTR4120 treatment

The ulcers were graded according to the NPUAP grading system. Table 1 shows that the ulcer grading in the treated and control groups reached their maximum at day 14. However, ulcers in

the OTR4120-treated group had significantly lower grades than in the control group ($p < 0.01$). Of the all ulcers (i.e. 64) graded on day 14, only 9 (14%) were superficial ulcers with intact skin (i.e. less than grade 2). Of those 9 ulcers, 8 (89%) were OTR4120-treated ulcers.

On day 21, 71% of ulcers were completed closed, 60% of which was treated with OTR4120.

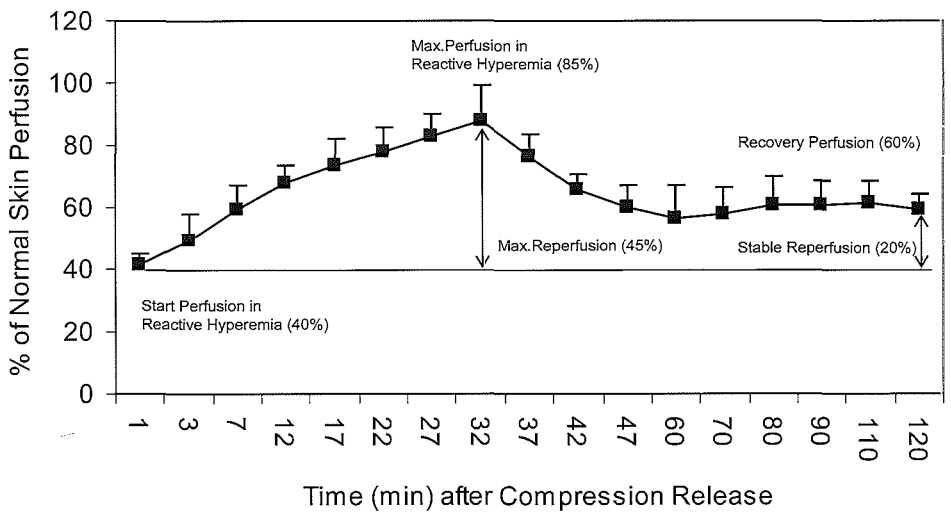

Figure 1. Change in perfusion of compressed skin following release of the magnets.

OTR4120 treatment reduced inflammation

Inflammation was evaluated by immunohistochemistry of the monocytes/macrophages in ulcer tissues using an anti-CD68 antibody (Figure 2). On days 3 and 14, CD68 staining was significantly reduced in the OTR4120-treated ulcers (2.62 ± 0.22 vs. 3.43 ± 0.18 on day 3, $p < 0.05$; and 2.98 ± 0.32 vs. 3.73 ± 0.24 on day 14, $p < 0.05$). No significant differences were found at any other time points. CD68 staining was not detectable on day 84. Western blot analysis also showed reduced CD68 expression in OTR4120-treated ulcers on day 14 (2.93 ± 0.97 vs. 9.48 ± 2.42, $p < 0.05$).

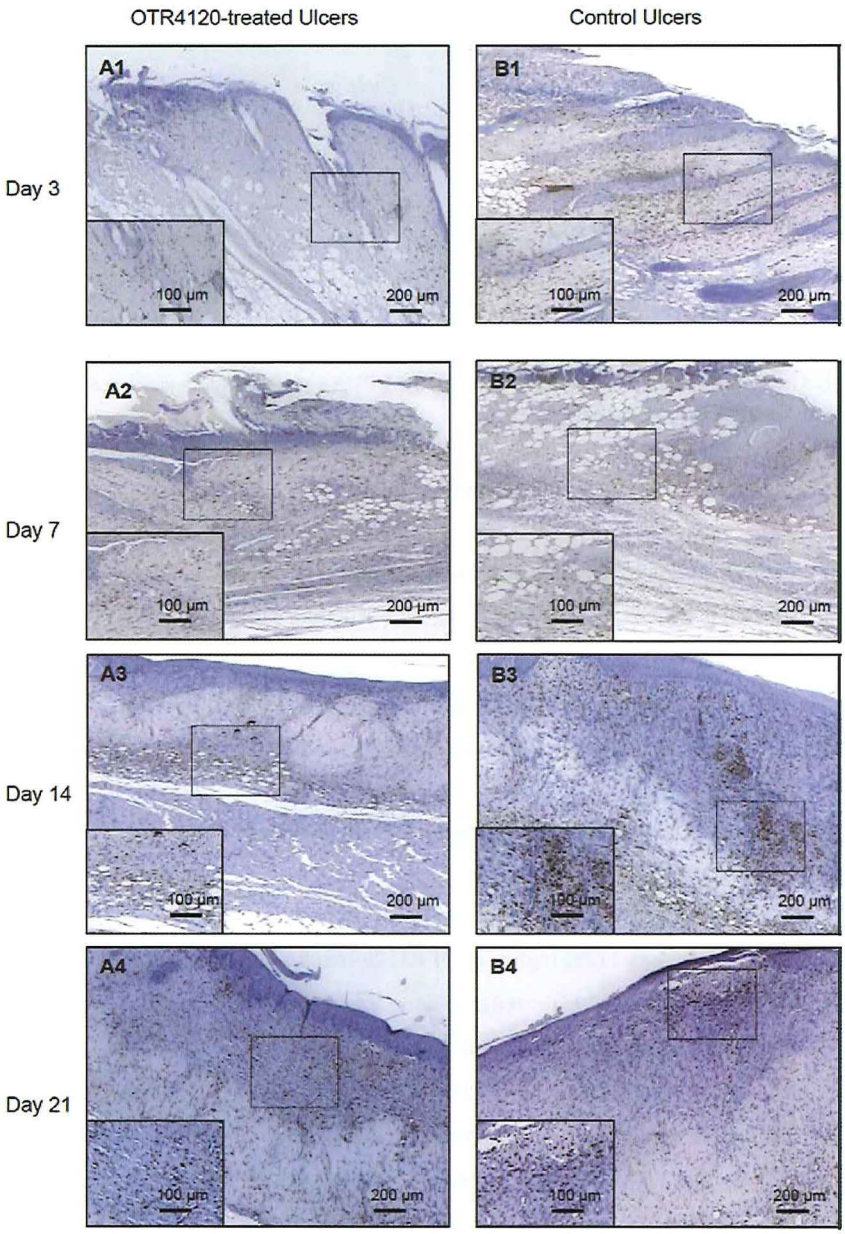

Figure 2. Effect of OTR4120 treatment on reducing inflammation assessed by monocyte/macrophage marker CD68 immunohistochemistry. Representative CD68-staining sections of OTR4120-treated ulcers (A1-4) and control ulcers (B1-4) on days 3, 7, 14, and 21 after compression release, respectively. CD68-staining was reduced in the OTR4120-treated ulcers on days 3 and 14. Original magnification × 40. Original inset magnification × 200.

Days after the removal of compression	Ulcer grade	
	OTR4120-treated	Control
1	1.26 ± 0.06 (n=8)	1.39 ± 0.05 (n=8)
3	1.57 ± 0.07 (n=16)	1.60 ± 0.05 (n=16)
7	1.59 ± 0.04 (n=32)	1.71 ± 0.09 (n=32)
14	2.75 ± 0.17 (n=32)*	3.28 ± 0.12 (n=32)*
21	0.55 ± 0.17 (n=16)	0.88 ± 0.19 (n=16)

Table 1. Grading of pressure ulcers according to the NPUAP pressure ulcer grading system. Data are presented as means ± SEM. *$p < 0.05$.

OTR4120 treatment stimulated angiogenesis

Angiogenesis was evaluated by immunohistochemistry of the endothelial cells in ulcer tissues using an anti-CD34 antibody (Figure 3). The CD34-staining score of OTR4120-treated ulcers was significantly higher than that of controls on days 14 and 21 (4.42 ± 0.33 vs. 3.57 ± 0.20 on day 14, $p < 0.05$; and 3.74 ± 0.28 vs. 2.72 ± 0.27 on day 21, $p < 0.01$). No significant differences were found at any other time points. CD34 staining was not detectable on day 84.

OTR4120 treatment increased the contents of angiogenic factors (i.e. VEGF and its receptor 1, TGF-β_1, and PDGF-A)

The presence of VEGF was 112% higher in OTR4120-treated ulcers than in the controls on day 14 (9.76 ± 4.13 vs. 4.60 ± 0.79, $p < 0.01$) (Figure 4A, F). VEGF receptor 1 (flt-1) was 63% higher in OTR4120-treated ulcers compared with the controls on day 3 (8.29 ± 0.77 vs. 5.07 ± 0.89, $p < 0.05$) and 223% higher on day 7 (7.91 ± 2.16 vs. 2.45 ± 0.04, $p < 0.05$) (Figure 4B, G). In OTR4120-treated ulcers the TGF-β_1 was 84% and 85% higher on day 3 and day 7, respectively, compared with the controls (16.84 ± 1.87 vs. 9.17 ± 1.20 on day 3, $p < 0.001$; and 9.41 ± 0.52 vs. 5.09 ± 0.50 on day 7, $p < 0.001$) (Figure 4C, H). In OTR4120-treated ulcers the PDGF-A was significantly higher compared with the controls during the first 14 days (11.59 ± 1.71 vs. 6.99 ± 0.45 on day 3, $p < 0.05$; 12.12 ± 2.56 vs. 3.73 ± 1.09 on day 7, $p < 0.05$; and 11.37

± 0.98 vs. 6.90 ± 0.77 on day 14, $p< 0.01$) (Figure 4D, I). All these factors were barely detectable on day 42.

OTR4120 treatment promoted early expression of alpha-smooth muscle actin

The expression of alpha-smooth muscle actin (α-SMA) was 109% higher in the OTR4120-treated ulcers than that in controls in the first week after release of compression (8.16 ± 1.00 vs. 3.90 ± 1.03 on day 3, $p< 0.05$, and 10.13 ± 1.43 vs. 5.95 ± 1.42 on day 7, $p< 0.01$) (Figure 4E, J). No difference was found between OTR4120-treated and non-treated groups on days 14 and 42.

OTR4120 treatment increased collagen biosynthesis

Collagen biosynthesis was assessed by determining the hydroxyproline content percentage, which was increased in the OTR4120-treated ulcers compared with the controls on day 21 (15.68 ± 1.02 μg hydroxyproline /mg homogenate vs. 11.62 ± 1.29 μg hydroxyproline /mg homogenate, $p< 0.05$).

OTR4120 treatment enhanced breaking strength

Restoration of ulcer tissue biomechanical strength was measured and expressed as the ratio of the ulcer breaking strength to that of the local normal skin tissue. This ratio in OTR4120-treated ulcers was 144% higher on day 42 and 58% higher on day 84 than those in the controls (0.66 ± 0.07 vs. 0.27 ± 0.03 on day 42, $p< 0.001$; 0.71 ± 0.05 vs. 0.45 ± 0.04 on day 84, $p< 0.01$) (Figure 5).

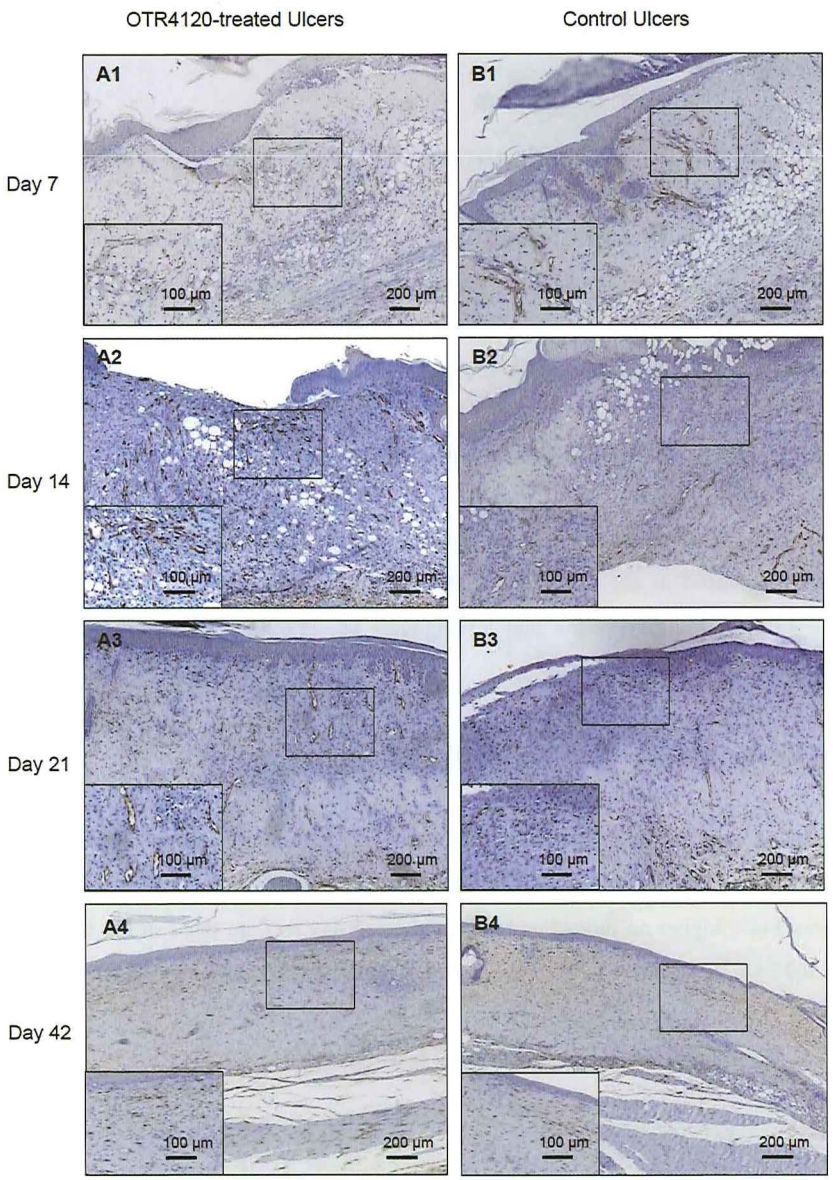

Figure 3. Effect of OTR4120 treatment on angiogenesis by the endothelial cell marker CD34 immunohistochemistry. Representative CD34-staining sections of OTR4120-treated ulcers (A1-4) and control ulcers (B1-4) on days 7, 14, 21, and 42 after compression release, respectively. OTR4120-treated ulcers had a significantly higher density of CD34 staining on days 14 and 21 than of the control ulcers. Original magnification × 40. Original inset magnification × 200.

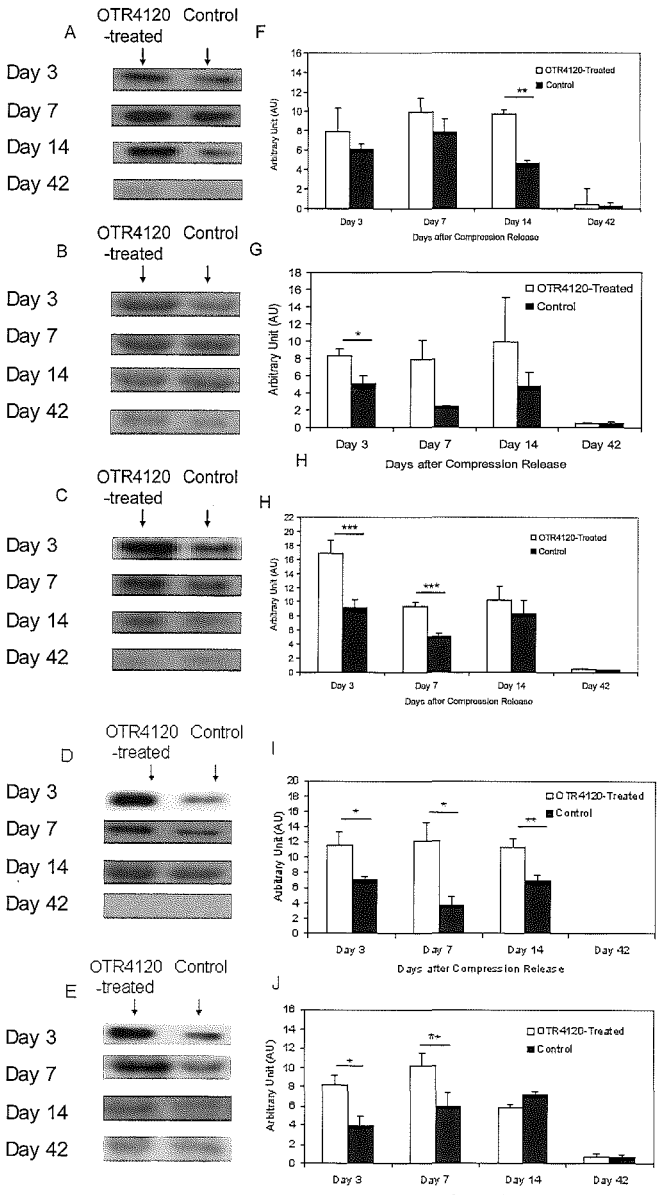

Figure 4. Effect of OTR4120 treatment on protein expressions assessed by Western blot analysis. Representative bands of VEGF (A), flt-1 (B), TGF-β1 (C), PDGF-A (D), and α-SMA (E) on days 3, 7, 14, and 42 after compression release, respectively. Quantification of the VEGF (F), flt-1 (G), TGF-β_1 (H), PDGF-A (I), and α-SMA bands (J). Data are presented as means ± SEM. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

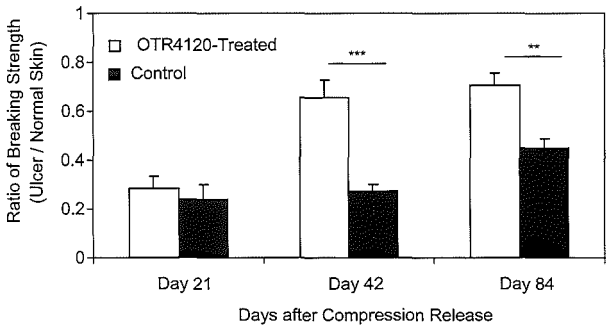

Figure 5. Ratio of the ulcer breaking strength to that of the normal skin tissue on days 21, 42, and 84. Data are presented as means ± SEM. ***$p < 0.001$ and **$p < 0.01$.

DISCUSSION

Pressure ulcers are clinically formed as consequence of patients adopting a fixed position for long periods of time. Parts of their bodies under constant pressure may develop ischemic conditions that can be subjected to rapid reperfusion upon positional changes [20]. Therefore, ischemia-reperfusion (I-R) injury is an important component of the etiology of pressure ulcers [21-23]. This clinical situation is mimicked in this model, in which at least 60% of normal skin perfusion was reduced during ischemic period, and at least 20% was reperfused to the ischemic region. This indicates that in this model, the auto regulatory blood flow mechanism is also capable of restoring skin perfusion. This mechanism can break down at a critical intensity and/or duration of pressure insult leaving only a single ischemic insult [24-25] whose etiology distinct from that of I-R injury [21].

As most pressure ulcers in patients result from a repetitive cycling of pressure and removal, Peirce and colleagues emphasize the number and the frequency of the cycles of I-R injury in animal ulceration models [22, 26]. In their model, a steel plate was implanted with repetitive magnet compressions for a total of 50 hours of ischemia time (2 hours/ischemia time, 5 cycles/day, 5 days) and created 36% necrotic area to the compressed area [22]. However, our

model of a single cycle of I-R injury with a successive 16-h ischemia period produced an equivalent 36% of skin necrosis on day 3 after compression removal. Therefore, we argue against the necessity of using the repetitive cycles of compression/removal to mimic clinical ulceration. Reid et al. also applied cyclical compression (similar to Peirce's model) in mice and found an equivalent degree of necrosis in mice that underwent more prolonged ischemic periods with minimal cycling. Reid et al. thus argued against the frequency of cycling of pressure and removal as the determining factor in ulceration [27]. A possible explanation for these findings is the adaptation mechanism that occurs when tissue is subjected to repetitive cycling of pressure and removal for long periods [28] , which is a common situation in patients with chronic wounds. For those patients, the protectively auto regulatory mechanisms can break down and reperfusion can gradually decrease and eventually disappear. Indeed, it is reported that debilitated patients are unable to respond to the repetitive pressure loading of the skin, even at comparatively low external pressures [29]. In addition, ethics of animal experimentation was also taken into account in this study, as the application of cyclical compressions increases stress on the experimental animals. In summary, we selected a single cycle I-R injury model because it was proved sufficient to create clinically graded ulcers. And also this model performed with a maximum attention for the welfare of the animals.

Consistent with clinical ulceration, in this model, edema, erythema, discoloration, induration, and final tissue necrosis were observed. Moreover, in this model, the animals were not anesthetized during the period of compression, thereby avoiding the confounding effect of anaesthesia on local tissue hemodynamics. It also supports the clinical relevance of this model.

I-R injury is considered as a significant mechanism in the early stages of pressure ulceration [23]. A continued influx of polymorphonuclear neutrophils (PMNs) and macrophages from circulation to inflammatory tissue contributes to the development of I-R injury [30]. PMNs can cause tissue damage in a variety of ways including secretion of proteolytic enzymes (e.g. neutrophil elastase and gelatinase) that lead to degradation of growth factors and structural proteins of ECM and secretion of free radical species (e.g. $^{\cdot}OH$, H_2O_2, and O_2^{-}) that can direct damage cells or the ECM [30-31]. Therefore, blockade of neutrophil–endothelial interactions is considered as one of the potential strategies to prevent I-R injury [30]. A variety of animal studies using anti-adhesion molecules antibodies have shown that the prevention of PMNs

infiltration can reduce reperfusion injury severity in several tissues. However, very few studies have been performed in the clinical setting due to the possibility of immune response following an intravenous administration of antibodies [30]. We found that OTR4120 had an anti-inflammation effect during ulceration. Although it remains unclear as to whether the reduced inflammation is specifically related to the I-R injury induced inflammation, it at least suggests that OTR4120 play an important role in facilitating an inflammation-controlled environment for subsequent progress through the normal stages of healing.

In addition to the highly proteolytic microenvironment induced by I-R injury, impaired angiogenesis is another characteristic of chronic wounds. In this model, although tissue hypoxia in the compressed area could induce compensatory angiogenesis [32], the consequent reperfusion injury may override the hypoxia-initiated angiogenesis and make it impaired. This may also explain why the angiogenic effect of OTR4120 was not observed in the first week of wound healing. The possible mechanism by which OTR4120 treatment stimulates angiogenesis is (at least partly) attributed by the increased presence of GFs (i.e. VEGF, TGF-β_1, and PDGF) observed in the present study. It should be noted that these increased GFs normalized to control levels in 3 weeks, suggesting that OTR4120 treatment allows the cellular tissue components to unfold their natural healing mechanisms, adapting to the internal needs to achieve tissue self-regeneration. This self-adjusted action of OTR4120 may be a key feature of OTR4120 treatment in improving the quality of wound healing.

α-SMA is transiently expressed by myofibroblasts during wound healing and participates in granulation tissue contractility both in vitro [33] and in vivo [34]. In this study, OTR4120-treated ulcer granulation tissue shows increased α-SMA expression level in the first week, suggesting that OTR4120 treatment may stimulate myofibroblast differentiation and early wound contraction. In addition to the activated fibroblasts in granulation tissue, another possible source of myofibroblasts are the vascular smooth muscle cells around the arterioles/venules; therefore, the increased α-SMA expression due to OTR4120 treatment may also favour the maturation of vessels and increase regional vasodilatory capability, thereby enhancing the protective auto-regulatory blood flow mechanisms and reducing the risk of developing pressure ulcers. This provides evidence for the current macroscopic finding that OTR4120 treatment reduced the degree of ulceration. The increase of TGF-β_1 and PDGF after OTR4120 treatment

may contribute to the mechanism in which OTR4120 treatment stimulates α-SMA expression because TGF-β_1, as the most accepted activator of fibroblasts, plays an important role in myofibroblast differentiation [35] and PDGF has a synergistic effect with TGF-β_1 in stimulating synthesis of fibroblasts, smooth muscle cells, collagen deposition and contraction [36-38].

Another important finding of this study is that OTR4120 treatment had a long-term effect on increasing ulcer biomechanical strength. Skin strength restoration is the down-stream phases of wound healing; hence, this could be affected by many events from the up-stream phases, however, collagen biosynthesis (especially cross-linked collagen biosynthesis) is an important element. The hydroxylation of proline residues is unique to collagen biosynthesis, and its degree will determine the number of cross-links and therefore the strength of the collagen [39]. The increased hydroxyproline content percentage in the presence of OTR4120 therefore contributes to increase of tissue strength. In addition, the increased presence of GFs, such as PDGF and TGF-β_1, also plays a role in improving breaking strength by stimulating fibroblast proliferation, differentiation, and ECM deposition [37]. The synergistic effects of the combination of TGF-β_1 and PDGF in enhancing tensile strength in rat incisional wounds have been reported earlier [37, 40].

In addition, OTR4120 treatment suppresses skin incomplete necrosis and, as a result, alleviates the degree of ulceration. It implies that OTR4120 treatment may have the potential to suppress I-R injury and prevent ulceration, which is of significant interest for ulcer management. However, the potential of OTR4120 treatment in preventing ulceration requires further investigations.

In spite of the date generated, the underlying mechanism of the therapeutic effect of OTR4120 is not specifically addressed by this study. Our next research plan is thus to investigate the mechanistic understanding to support the biological properties of OTR4120 in preventing ulceration and improving ulcer healing.

In summary, we demonstrate that OTR4120 treatment improves I-R injury-induced pressure ulcer healing by reducing inflammation, stimulating angiogenesis, increasing collagen biosynthesis, and biomechanical strength. Our findings suggest that OTR4120 is a potent regenerative agent for the treatment of pressure ulcers.

ACKNOWLEDGEMENTS

The authors thank Prof. D.W. van Bekkum (Leiden University Medical Centre, Leiden, the Nethelands) for his assistance in the development of the pressure ulcer model, and Prof. D. Barritault (OTR3, SAS, Paris, France) for providing OTR4120. The authors also thank Dr. S. P. Willemsen (Department of Biostatistics, Erasmus MC, University Medical Center, Rotterdam, the Netherlands) for his consultation on statistical analysis. This research was supported by a grant from the Nuts Ohra Foundation (the Netherlands), grant No. SNO-T-0-0501-159.

Partial content of this paper has been presented in the 12th European Conference of Scientists and Plastic Surgeons congress in Bern, Switzerland, 2008, with its abstract being pressed in Journal of Plastic Reconstructive and Aesthetic Surgery 2009; 834-35.

REFERENCES

1. Lyder, C.H., Pressure ulcer prevention and management. Jama, 2003. **289**(2): p. 223-6.
2. Lardenoye, J.W., J.A. Thiefaine, and P.J. Breslau, Assessment of incidence, cause, and consequences of pressure ulcers to evaluate quality of provided care. Dermatol Surg, 2009. **35**(11): p. 1797-803.
3. Bansal, C., et al., Decubitus ulcers: a review of the literature. Int J Dermatol, 2005. **44**(10): p. 805-10.
4. Bello, Y.M. and T.J. Phillips, Recent advances in wound healing. Jama, 2000. **283**(6): p. 716-8.
5. Dini, V., M. Bertone, and M. Romanelli, Prevention and management of pressure ulcers. Dermatol Ther, 2006. **19**(6): p. 356-64.
6. Stadelmann, W.K., A.G. Digenis, and G.R. Tobin, Physiology and healing dynamics of chronic cutaneous wounds. Am J Surg, 1998. **176**(2A Suppl): p. 26S-38S.
7. Salcido, R., A. Popescu, and C. Ahn, Animal models in pressure ulcer research. J Spinal Cord Med, 2007. **30**(2): p. 107-16.
8. Bosman, F.T. and I. Stamenkovic, Functional structure and composition of the extracellular matrix. J Pathol, 2003. **200**(4): p. 423-8.
9. Hacker, U., K. Nybakken, and N. Perrimon, Heparan sulphate proteoglycans: the sweet side of development. Nat Rev Mol Cell Biol, 2005. **6**(7): p. 530-41.
10. Palolahti, M., et al., Proteolytic activity in leg ulcer exudate. Exp Dermatol, 1993. **2**(1): p. 29-37.
11. Lauer, G., et al., Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. J Invest Dermatol, 2000. **115**(1): p. 12-8.
12. Menke, N.B., et al., Impaired wound healing. Clin Dermatol, 2007. **25**(1): p. 19-25.
13. Roth, D., et al., Plasmin modulates vascular endothelial growth factor-A-mediated angiogenesis during wound repair. Am J Pathol, 2006. **168**(2): p. 670-84.
14. Lauer, G., et al., Generation of a novel proteolysis resistant vascular endothelial growth factor165 variant by a site-directed mutation at the plasmin sensitive cleavage site. FEBS Lett, 2002. **531**(2): p. 309-13.
15. Tong, M., et al., RGTA OTR 4120, a heparan sulfate proteoglycan mimetic, increases wound breaking strength and vasodilatory capability in healing rat full-thickness excisional wounds. Wound Repair Regen, 2008. **16**(2): p. 294-9.
16. Tong, M., et al., Stimulated neovascularization, inflammation resolution and collagen maturation in healing rat cutaneous wounds by a heparan sulfate glycosaminoglycan mimetic, OTR4120. Wound Repair Regen, 2009. **17**(6): p. 840-52.

17. Stadler, I., et al., Development of a simple, noninvasive, clinically relevant model of pressure ulcers in the mouse. J Invest Surg, 2004. **17**(4): p. 221-7.
18. Barbosa, I., et al., A synthetic glycosaminoglycan mimetic (RGTA) modifies natural glycosaminoglycan species during myogenesis. J Cell Sci, 2005. **118**(Pt 1): p. 253-64.
19. Woessner, J.F., Jr., The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys, 1961. **93**: p. 440-7.
20. Salcido, R., et al., Histopathology of pressure ulcers as a result of sequential computer-controlled pressure sessions in a fuzzy rat model. Adv Wound Care, 1994. **7**(5): p. 23-4, 26, 28 passim.
21. Tsuji, S., et al., Analysis of ischemia-reperfusion injury in a microcirculatory model of pressure ulcers. Wound Repair Regen, 2005. **13**(2): p. 209-15.
22. Peirce, S.M., T.C. Skalak, and G.T. Rodeheaver, Ischemia-reperfusion injury in chronic pressure ulcer formation: a skin model in the rat. Wound Repair Regen, 2000. **8**(1): p. 68-76.
23. Jiang, L.P., et al., Ischemia-reperfusion injury-induced histological changes affecting early stage pressure ulcer development in a rat model. Ostomy Wound Manage. **57**(2): p. 55-60.
24. Capp, C.L., et al., Post pressure hyperemia in the rat. Comp Biochem Physiol A Mol Integr Physiol, 2004. **137**(3): p. 533-46.
25. Kvernebo, K., C.E. Slasgsvold, and E. Stranden, Laser Doppler flowmetry in evaluation of skin post-ischaemic reactive hyperaemia. A study in healthy volunteers and atherosclerotic patients. J Cardiovasc Surg (Torino), 1989. **30**(1): p. 70-5.
26. Saito, Y., et al., The loss of MCP-1 attenuates cutaneous ischemia-reperfusion injury in a mouse model of pressure ulcer. J Invest Dermatol, 2008. **128**(7): p. 1838-51.
27. Reid, R.R., et al., A novel murine model of cyclical cutaneous ischemia-reperfusion injury. J Surg Res, 2004. **116**(1): p. 172-80.
28. Goldstein, B. and J. Sanders, Skin response to repetitive mechanical stress: a new experimental model in pig. Arch Phys Med Rehabil, 1998. **79**(3): p. 265-72.
29. Bader, D.L., The recovery characteristics of soft tissues following repeated loading. J Rehabil Res Dev, 1990. **27**(2): p. 141-50.
30. Anaya-Prado, R., et al., Ischemia/reperfusion injury. J Surg Res, 2002. **105**(2): p. 248-58.
31. Vajdovich, P., Free radicals and antioxidants in inflammatory processes and ischemia-reperfusion injury. Vet Clin North Am Small Anim Pract, 2008. **38**(1): p. 31-123, v.
32. Shweiki, D., et al., Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature, 1992. **359**(6398): p. 843-5.
33. Hinz, B., et al., Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. Mol Biol Cell, 2001. **12**(9): p. 2730-41.

34. Hinz, B., et al., Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. Am J Pathol, 2001. **159**(3): p. 1009-20.
35. Desmouliere, A., C. Chaponnier, and G. Gabbiani, Tissue repair, contraction, and the myofibroblast. Wound Repair Regen, 2005. **13**(1): p. 7-12.
36. Tung, P.S. and I.B. Fritz, Transforming growth factor-beta and platelet-derived growth factor synergistically stimulate contraction by testicular peritubular cells in culture in serum-free medium. J Cell Physiol, 1991. **146**(3): p. 386-93.
37. Ashraf, A., et al., Effect of sustained-release PDGF and TGF-beta on cyclophosphamide-induced impaired wound healing. Plast Reconstr Surg, 2009. **124**(4): p. 1118-24.
38. Lynch, S.E., et al., Role of platelet-derived growth factor in wound healing: synergistic effects with other growth factors. Proc Natl Acad Sci U S A, 1987. **84**(21): p. 7696-700.
39. Ghosh, A.K., Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. Exp Biol Med (Maywood), 2002. **227**(5): p. 301-14.
40. Hosgood, G., Wound healing. The role of platelet-derived growth factor and transforming growth factor beta. Vet Surg, 1993. **22**(6): p. 490-5.

CHAPTER 5

Diabetes-impaired wound healing is improved by matrix therapy with heparan sulfate glycosaminoglycan mimetic OTR4120 in rats

Miao Tong, Bastiaan Tuk, Peng Shang, Ineke M. Hekking, Esther M.G. Fijneman, Marnix Guijt, Steven E.R. Hovius, Johan W. van Neck

Diabetes 2012; 61: 2633-2641

ABSTRACT

Wound healing in diabetes is frequently impaired and its treatment remains a challenge. We tested a therapeutic strategy of potentiating intrinsic tissue regeneration by restoring the wound cellular environment using a heparan sulfate glycosaminoglycan mimetic, OTR4120. The effect of OTR4120 on healing of diabetic ulcers was investigated. Experimental diabetes was induced by intraperitoneal injection of streptozotocin. Seven weeks after induction of diabetes, rats were ulcerated by clamping a pair of magnet disks on the dorsal skin for 16 h. After magnet removal, OTR4120 was administered via an intramuscular injection weekly for up to 4 weeks. To examine the effect of OTR4120 treatment on wound healing, the degree of ulceration, inflammation, angiogenesis, and collagen synthesis were evaluated. We found that OTR4120 treatment significantly reduced the degree of ulceration and the time of healing. These effects were associated with reduced neutrophil infiltration and macrophage accumulation, and enhanced angiogenesis. OTR4120 treatment also increased the collagen content with an increase of collagen type I biosynthesis and reduction of collagen type III biosynthesis. Moreover, restoration of the ulcer biomechanical strength was significantly enhanced following OTR4120 treatment. This study shows that matrix therapy with OTR4120 improves diabetes-impaired wound healing.

INTRODUCTION

Impaired wound healing is a well-documented phenomenon both in experimental and clinical diabetes [1]. Several mechanisms for diabetes-impaired wound healing are proposed that are mostly related to impairment of macrophage function [2], angiogenic response [3], and extracellular matrix (ECM) deposition [4]. The ideal treatment relies on correcting the multiple deficits simultaneously through highly integrated and personalized therapeutic approaches.

Wound healing is associated with dynamic interactions between the ECM and growth factors (GFs) [5]. The ECM consists of a network of scaffold proteins that are bridged by glycosaminoglycans (GAGs), of which heparan sulfate (HS) is an important component. HS-GAGs are capable of transmitting signals by providing binding sites for a large variety of HS-bound signaling peptides (i.e. GFs, chemokines, and cytokines). However, following tissue injury, the glycanases and proteases can destroy HS-GAGs [6]. As a result, the ECM-GFs interactions are disturbed. These disruptions characterize impaired wounds [5, 7] and also may have the implication of matrix therapy for wound treatment.

OTR4120 is a HS-GAG mimetic that can replace the degraded HS-GAGs, and protect and improve the bioavailability of GFs, cytokines and other heparin-binding signaling peptides. In this way, OTR4120 offers a matrix therapy that restores the natural cellular microenvironment and the endogenous signaling of cell communications needed for tissue regeneration [8-10]. This facilitates the quality of wound healing by potentiating the intrinsic tissue regeneration.

The current study evaluates the efficacy of matrix therapy with OTR4120 in pressure ulcers generated in streptozotocin (STZ)-induced diabetic rats.

RESEARCH DESIGN AND METHODS

Animals

WAG/RijHsd female rats (142, 10 weeks old) were purchased from Harlan (Zeist, the Netherlands). Rats were exposed to a 12 h light/dark cycle and fed a standard laboratory diet with food and water available ad libitum. All procedures with animals were approved by the local Animal Experiments Committee.

Induction of diabetes mellitus

After overnight fasting, animals were given an intraperitoneal injection of STZ (Sigma-Aldrich, St. Louis, MO) at a dose of 65 mg/kg bodyweight in 0.05 mol/L sodium citrate buffer, pH 4.5. Blood glucose concentration was monitored weekly by an OneTouch Glucometer (LifeScan, Milpitas, CA) from tail vein blood. A prolonged diabetic status was defined as blood glucose levels $\geqslant$ 20 mmol/L throughout the induction period.

Ulceration model and OTR4120 treatment

Seven weeks after STZ injection, 119 diabetic rats were ulcerated by clamping and then removal of a pair of magnet disks (15 mm diameter) on rat dorsal skin for a single ischemic period of 16 h. After wounding, rats were randomly allocated to 6 groups to serve 6 experimental end points (i.e. day 3, n=16; day 7, n=18; day 14, n=18; day 18, n=15; day 42, n=34, and day 84, n=18). Lyophilized OTR4120 was rehydrated in a physiological salt solution (B. Braun Melsungen AG, Melsungen, Germany) at a concentration of 1mg/mL. Immediately after magnet removal, rats were randomly assigned to receive an intramuscular injection of OTR4120 in the thigh at a dose of 1 mg/kg bodyweight or the same volume of physiological salt solution weekly for up to 1 month. The OTR4120 dosage was based on the experience from the previous studies [11-15]. At each experimental end time point, the animals were killed simultaneously. The experiment was blinded to all observers.

Macroscopic analysis

Bodyweight and blood glucose levels were measured and the ulcers were photographed. The ulcers were graded according to the grading system of the National Pressure Ulcer Advisory Panel [16]. The percentage of completely closed ulcers was calculated.

Immunohistochemistry

Paraffin-embedded sections (5 um) were deparaffinized and rehydrated. Antigen retrieval was performed in Tris-EDTA (TE) buffer containing 0.1% trypsin (Invitrogen, Carlsbad, CA). Endogenous peroxidase activity was quenched by exposing to 0.1% hydrogen peroxide in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST). After blocking with 4% non-fat milk powder in PBST, the sections were incubated with mouse anti-CD68 (1: 100; AbD Serotec, Düsseldorf, Germany) and goat anti-CD34 (1:200; R&D Systems, Minneapolis, MN), respectively, and then followed by incubating with the corresponding biotinylated secondary antibodies (R&D systems). The antigen-antibody complex was detected by streptavidin-peroxidase (R&D systems) and 3,3-diaminobenzidine (Dako, Carpinteria, CA). For evaluation of staining, the overview of the positive-signal density was scored semi quantitatively as 1 (absent), 2 (low), 3 (medium), 4 (strong), and 5 (very strong). The median of scores from three observers, who were blinded to the treatments, was used for comparisons.

Myeloperoxidase assay

Myeloperoxidase (MPO) activity in ulcer tissue homogenates was assayed as previously described with minor modifications [17]. Briefly, homogenates were mixed in 100mmol/L sodium acetate buffer containing 0.5% hexadecyl trimethyl ammonium bromide (Sigma-Aldrich). The mixture was centrifuged to extract the MPO. After centrifugation, the supernatant was incubated at 37 °C for 15 min in 100 mmol/L sodium acetate buffer, containing 0.0005% hydrogen peroxide, 3.2 mmol/ 3,3′ , 5,5′ -tetramethylbenzidine dihydrochloride (Sigma-Aldrich), and 0.5% hexadecyl trimethyl ammonium bromide. The optical densities were

recorded at 660 nm. MPO content was determined as units per gram tissue homogenate by using a standard curve generated by purified MPO from human leukocytes (Sigma-Aldrich).

Western blot analysis

Protein contents of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor beta-1 (TGF-β_1), and inducible nitric oxide synthase (iNOS) in ulcer tissue homogenates were evaluated by Western blot as described previously [13]. In brief, the protein concentrations of homogenates were determined using RcDc protein assay kit (Bio-Rad, Hercules, CA). An equivalent amount of protein was separated on an SDS polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). Blocking was performed in 3% bovine serum albumin (Sigma-Aldrich) in 10 mmol/L Tris, 150 mmol/L NaCl, containing 0.1% Tween-20. The membranes were probed with 1:1000 diluted mouse anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), 1: 400 diluted rabbit anti-PDGF (Santa Cruz Biotechnology), 1:500 diluted mouse anti-TGF-β1 (R&D Systems), and 1:400 diluted rabbit anti-iNOS (Calbiochem, San Diego, CA), respectively. Binding of the primary antibody was detected using a peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). Positive bands were visualized using chemiluminescence (Supersignal®, Pierce).

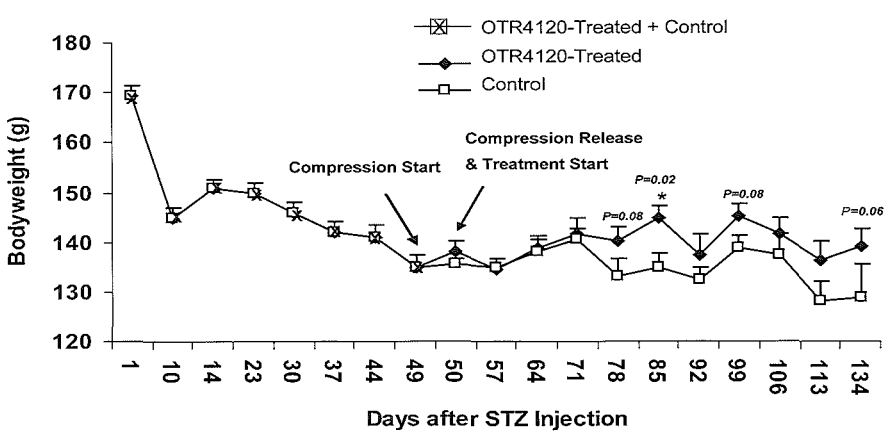

Figure 1. Bodyweight changes during the periods of STZ-diabetes induction (i.e. days1 to 49 after STZ injection), and ulceration and healing (i.e. days 49 to134). Data are mean ± SEM. * $p < 0.05$ indicates significant difference of the weight restoration following STZ injection between the OTR4120-treated rats and control diabetic rats.

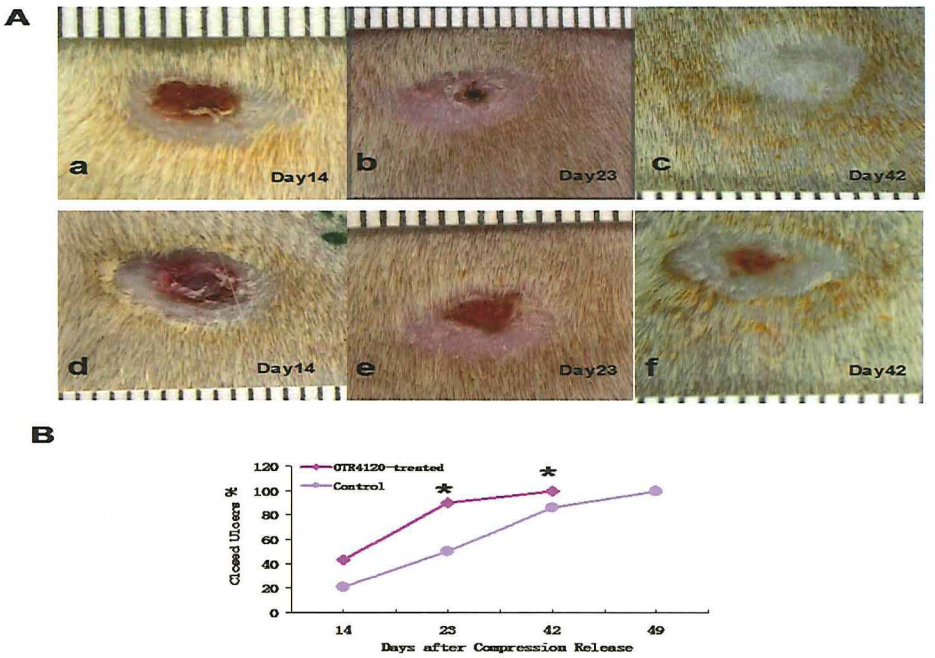

Figure 2. Macroscopic evaluation of the ulcer healing in OTR4120-treated and control rats. Representative photographs (A) of ulcers in OTR4120-treated rats on day 14 (a), day 23 (b), and day 42 (c), and in control rats on day 14 (d), day 23 (e), and day 42 (f). Percentage of complete closed ulcers (B). * $p < 0.05$ indicates significant difference between the OTR4120-treated rats and control rats.

Hydroxyproline content and collagen type I, type III ex vivo biosynthesis measurements

Ulcer biopsies were labelled with 50 mCi (^{3}H) hydroxyproline (GE Healthcare, Diegem, Belgium) for 24 h in Dulbecco's modified Eagle's minimal essential medium supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mmol/L glutamine. The tissue was then washed extensively until no radioactivity was detected. Subsequently, the labelled biopsies were homogenized and a small aliquot of the homogenates was hydrolyzed in 6 N HCl at 100°C for 24 h. The total collagen content was determined by colorimetric hydroxyproline assay [18]. All remaining homogenates were digested with 1% pepsin (Sigma-Aldrich) in 0.5 M acetic acid for 48 h. After centrifugation, the supernatant was dialyzed against 0.5 M acetic acid for 24 h and

then lyophilized. Before lyophilization, the total pepsin-soluble biosynthesized collagen was measured by determining the amount of (^{3}H) hydroxyproline in the dialysates. Biosynthesis of collagen type I and type III was determined following electrophoresis of a 1-mg lyophilized sample on SDS-PAGE. The collagen bands were revealed by Coomassie Brilliant Blue staining and identified by comparison with standard collagen type I and type III. The relative proportions of radioactivity incorporated in collagen types were quantified by excision of each individual collagen band followed by hydrolysis of the band in 6N HCl at 100°C for 24 h, after which (^{3}H) hydroxyproline was determined in the hydrolysate [11].

Breaking strength measurement

Breaking strength was measured as described previously [12]. In brief, the excised dorsal pelt, containing an ulcer was cut into two standardized dumbbell-shaped skin strips. One strip was centred by a segment of ulcer, and the other was cut from the surrounding normal skin. The strip was fixed perpendicularly between the two clips of a tensiometer and subjected to a constant strain rate of 60mm/min using a 1.0kg force transducer. Breaking strength was recorded as the maximum load (Newton) measured before skin failure. The ratio of ulcer breaking strength to that of surrounding normal skin breaking strength was calculated for data analysis.

Real-time quantitative PCR

Total RNA from tissues was isolated using TRIzol reagent (Invitrogen). Five micrograms of total RNA was treated for 15 min with DNase I prior to a 50 min reverse transcription reaction using a SuperScript II RT kit (Invitrogen). Quantitative PCR was performed with a SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) using a C1000 Thermal Cycler (BIO-RAD). Melting curves were used as quality controls to exclude samples with genomic DNA content and to ensure single product amplification. Beta-actin levels were used for internal normalization. Data were analyzed with the CFX Manager 2.1 software package (Bio-Rad). The primer sets are described as follows: VEGF-A, Forward 5'-ACGTCACTATGCAGATCATGC-3', Reverse 5'-CCTTTCCCTTTCCTCGAACTG-3'; TGF-β1, Forward 5' CAATTCCTG GCGTTACCTTG3', Reverse 5' AAAGCCCTGTATTCCGTCTC3'; iNOS, Forward 5'-GAACTCGGGCATACCTTCAG-3', Reverse 5'-CTCCCAGGTGAGACAGTTTC-3'.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Statistical calculations were performed using SPSS software, version 11, (Chicago, IL). The Mann-Whitney U-test was carried out to compare results between groups. A p-value ≤ 0.05 was considered to indicate a statistically significant difference.

RESULTS

Diabetes induction

During the 7-week diabetes induction period, 84% of the STZ-injected rats became consistently hyperglycemic and were included in this study. Of the STZ-injected rats, 7% had glucose levels $\leq$ 20 mmol/L, 5% died, and 4% were killed because of a body weight loss of $\geq$ 10 g/week.

Bodyweight changes

During the diabetes induction period the rats had a weight reduction of up to 17% at the day 10 after STZ injection (Figure 1). Following STZ injection, from 78 days onwards, the average weight restoration was, almost significantly, higher in the OTR4120-treated rats than in control rats (87% vs. 80% on day 78, p=0.08; 90% vs. 81% on day 85, p=0.02; 91% vs. 83% on day 99, p=0.08; and 87% vs. 77% on day 134, p=0.06) (Figure 1).

OTR4120 treatment reduced the degree of ulceration

OTR4120-treated ulcers had significantly lower National Pressure Ulcer Advisory Panel grades compared with control ulcers on day 14 (1.68 ± 0.23 vs. 2.50 ± 0.25, $p < 0.05$).

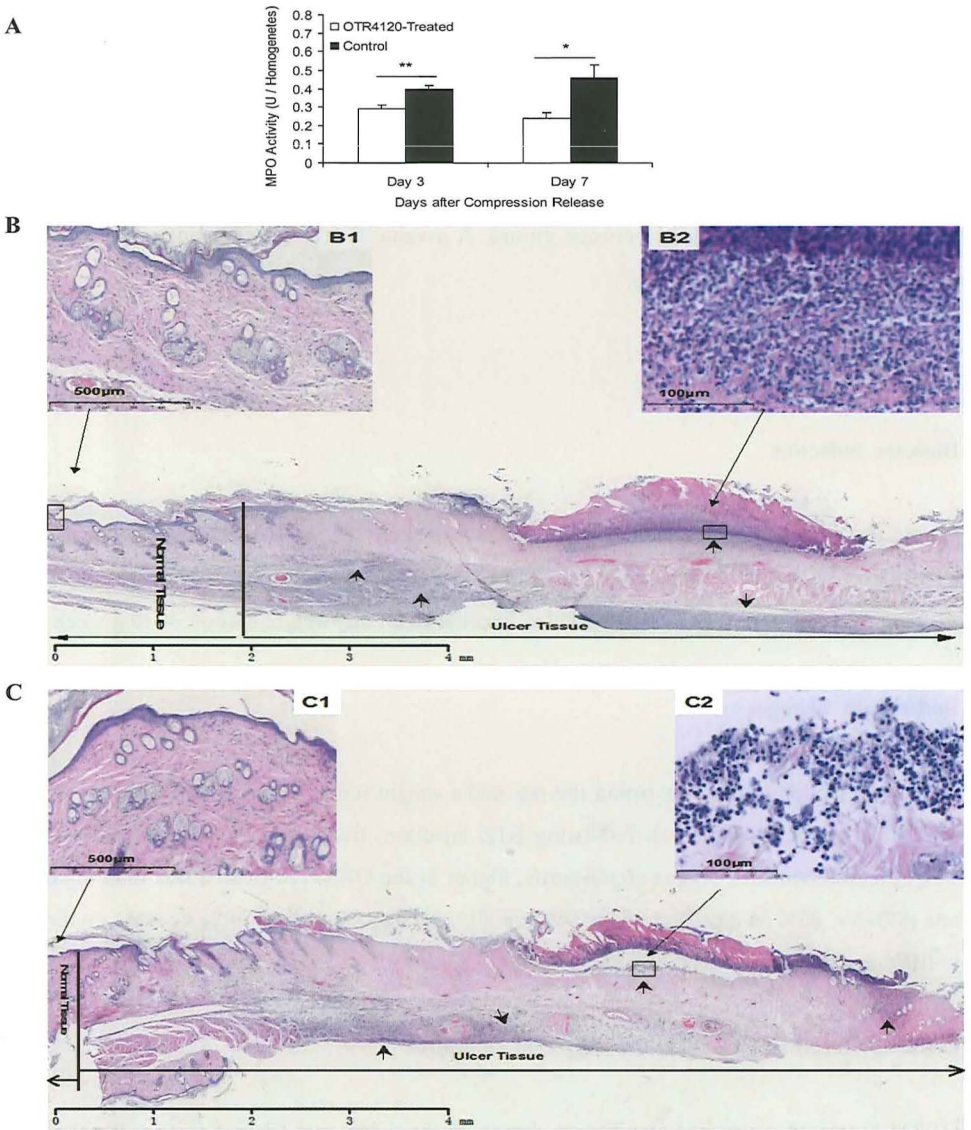

Figure 3. The effect of OTR4120 treatment on reducing neutrophils infiltration assessed by MPO activity assay (A) and H&E stained histology (B and C). Representative H&E staining section on day 7 after compression release of a control ulcer (B) shows intense infiltration of neutrophils (arrows) (original magnification x 1.25; scale bar = 4 mm) in the ulcer area, and an OTR4120-treated ulcer (C) shows less intense infiltration of neutrophils (arrows) (original magnification x 1.25; scale bar = 4 mm). B and C, the top left inserts: Normal skin tissues, located 2 mm from the ulcer margin of a control ulcer (B1) and an OTR4120-treated ulcer (C1) (original magnification x 10; scale bar = 500µm). B and C, the top right inserts: Neutrophil infiltration at higher magnification of a control ulcer (B2) and an OTR4120-treated ulcer (C2) (original magnification x 40; scale bar = 100µm). Data are presented as means ± SEM. $*p < 0.05$ and $**p < 0.01$ indicate significant differences between the OTR4120-treated groups and control groups.

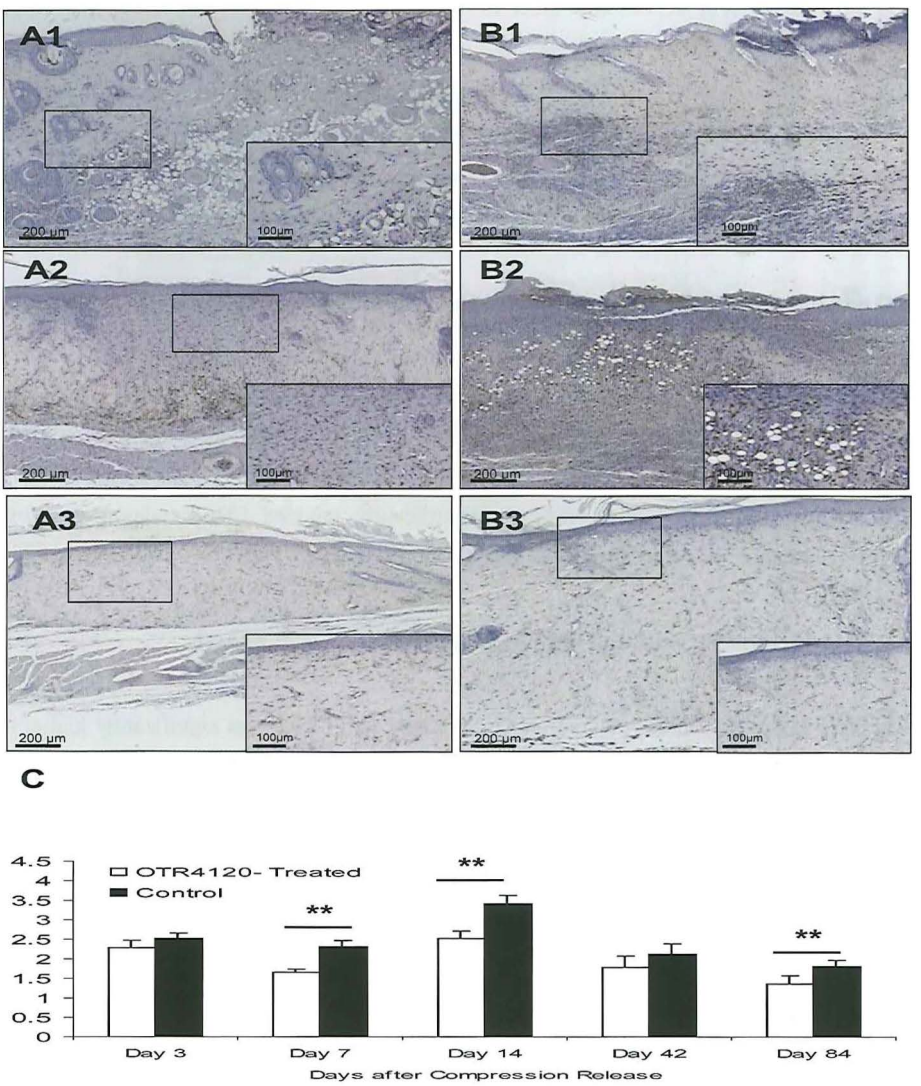

Figure 4. The effect of OTR4120 treatment on inflammation reduction assessed by monocyte/macrophage marker CD68 immunohistochemistry. Representative CD68-staining sections of OTR4120-treated ulcers (A1-A3) and control ulcers (B1-B3) on days 7, 14, and 84 after compression release, respectively. Original magnification x 40. Original Inset magnification x 200. Graphic visualization of scores of CD68 staining at indicated time points (C). Data are presented as means ± SEM. $^{**}p < 0.01$ indicates significant difference between the OTR4120-treated groups and control groups.

OTR4120 treatment accelerated ulcer healing

Figure 2 shows that 90% of OTR4120-treated ulcers were completely closed on day 23 after compression release, compared with 50% of control ulcers (p <0.05). All OTR4120-treated ulcers were completely closed on day 42, whereas 14% of control ulcers were still open (p <0.05). All control ulcers were completely closed on day 49 (data not shown).

OTR4120 treatment reduced neutrophil infiltration and stimulated inflammation resolution

MPO activity was 28% lower in the OTR4120-treated rats compared with control rats on day 3 ($p < 0.01$), and 48% lower on day 7 ($p < 0.05$) (Figure 3). CD68-staining was detectable during the entire observation period. However, significantly reduced CD68 staining was found in OTR4120-treated ulcers on days 7, 14 and 84 (Figure 4).

OTR4120 treatment enhanced nerovascularization and angiogenesis

The density score of CD34 staining in OTR4120-treated ulcers was significantly higher than in controls on day 14 (3.85 ± 0.22 vs. 2.94 ± 0.21, $p < 0.01$) and on day 42 (2.99 ± 0.14 vs. 2.41 ± 0.16, $p < 0.01$) (Figure 5). The presence of VEGF was 197% higher in OTR4120-treated ulcers than in controls on day 14 ($p < 0.05$) (Figure 6A and D). TGF-β1 was 72% higher in OTR4120-treated ulcers than in controls on day 3 ($p < 0.01$) and 40% higher on day 14 ($p < 0.01$) (Figure 6B and E). All scores had reduced to control levels on day 84.

OTR4120 treatment revealed more iNOS

In OTR4120-treated ulcers the presence of iNOS was 66% higher on day 3 (4.04 ± 0.46 vs. 2.43 ± 0.52, $p < 0.05$) and 88% higher on day 14 (5.40 ± 0.87 vs. 2.87 ± 0.35, $p < 0.05$) compared with controls (Figure 6C and F).

OTR4120 treatment enhanced breaking strength

The skin strength ratio in OTR4120-treated ulcers was 160% higher on day 18, 33% higher on day 42, and 33% higher on day 84 compared with controls (Figure 7).

OTR4120 treatment increased hydroxyproline content

The hydroxyproline content was increased in OTR4120-treated ulcers compared with controls on day 7 (9.81 ± 0.34 vs. 6.89 ± 0.42 μg hydroxyproline /mg homogenate, $p < 0.05$), and on day 42 (14.31 ± 1.11 μg vs. 10.18 ± 1.01 μg hydroxyproline /mg homogenate, $p < 0.05$).

OTR4120 treatment increased ex vivo biosynthesis of collagen type I and reduced collagen type III

The percentage of synthesized type I collagen was significantly increased in OTR4120-treated ulcers on day 42 (92.1 ± 1.36 vs. 86.9 ± 1.57, $p < 0.05$) (Figure 8A). In contrast, collagen type III synthesis was reduced in OTR4120-treated ulcers on day 7 and day 42 (9.1 ± 1.00 vs. 12.4 ± 1.10 on day 7, $p = 0.07$; 4.86 ± 1.26 vs. 9.75 ± 1.47 on day 42, $p < 0.05$) (Figure 8B). The ratio of collagen type I to type III in the OTR4120-treated ulcers was increased twofold compared with control ulcers on day 42 (32.8 ± 7.43 vs. 11.57 ± 3.07, $p < 0.05$) (Figure 8C).

OTR4120 treatment had no effect on VEGF-A, TGF-β1, and iNOS gene transcription

No statistical differences in VEGF-A, TGF-β1, and iNOS gene transcription were found between OTR4120-treated and control groups.

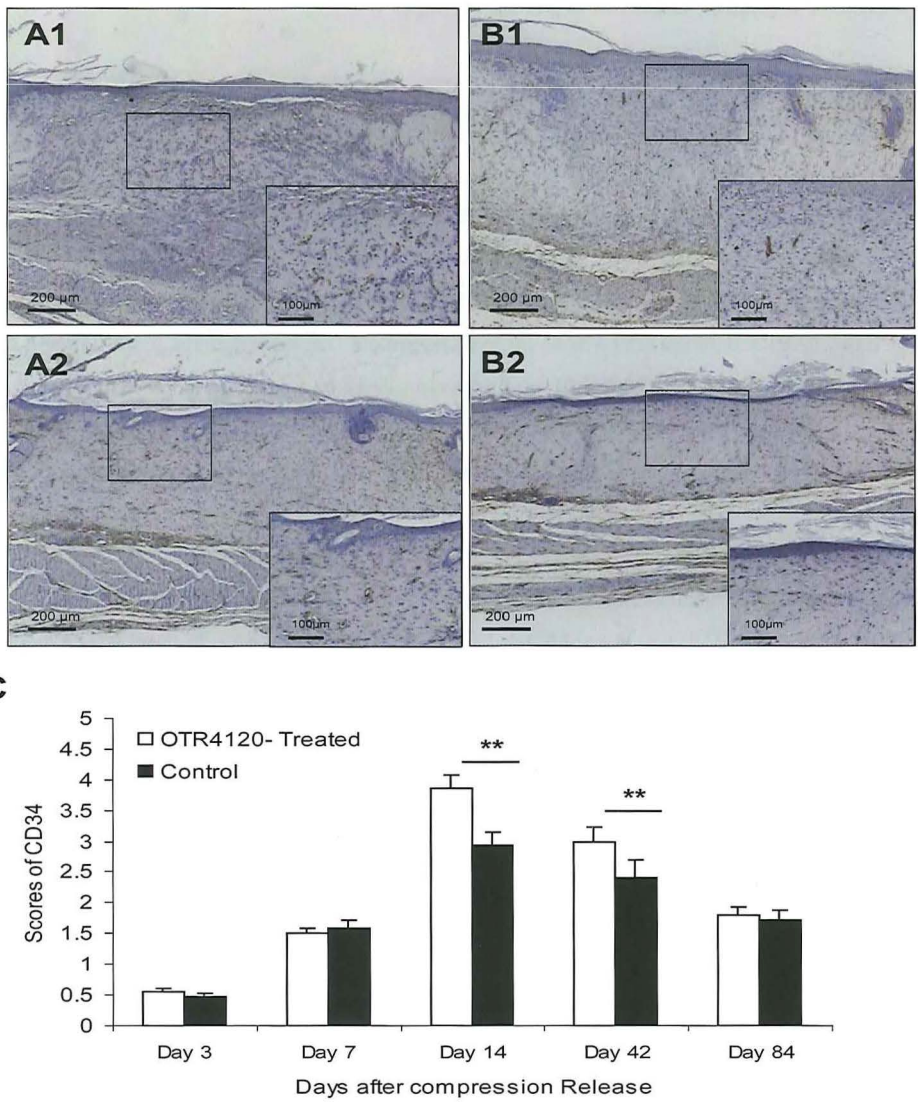

Figure 5. The effect of OTR4120 treatment on enhancing nerovascularization assessed by the endothelial cell marker CD34 immunohistochemistry. Representative CD34-staining sections of OTR4120-treated wounds (A1-A2) and control wounds (B1-B2) on days 14 and 42 after compression release, respectively. Original magnification x 40. Original inset magnification x 200. Graphic visualization of scores of CD34 staining at indicated time points (C). Data are presented as means ± SEM. $^{**}p < 0.01$ indicates significant difference between the OTR4120-treated groups and control groups.

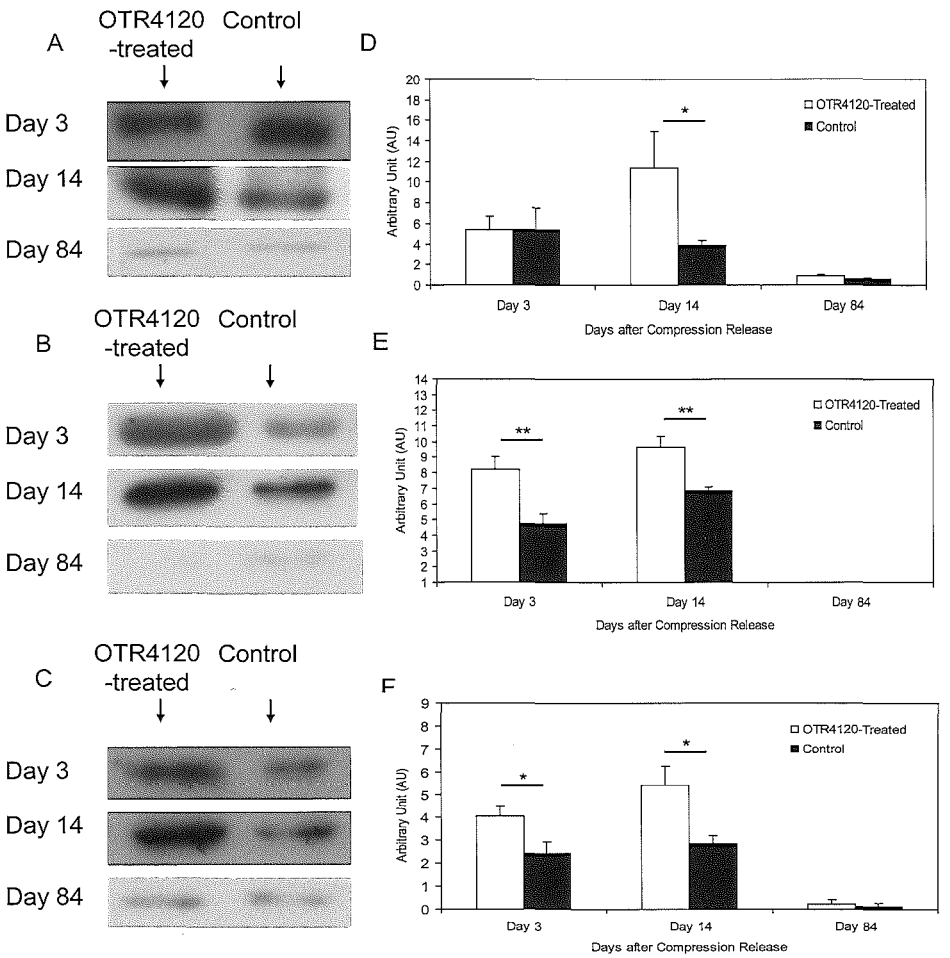

Figure 6. The effect of OTR4120 treatment on increasing the contents of VEGF, TGF-β1, and iNOS as assessed by Western blot analysis. Representative bands of VEGF (A), TGF-β1 (B), and iNOS (C) on days 3, 14, and 84 after compression release, respectively. Quantification of the VEGF (D), TGF-β1 (E), and iNOS bands (F). Data are presented as means ± SEM. $*p < 0.05$, and $**p < 0.01$ indicate significant differences between the OTR4120-treated rats and control rats.

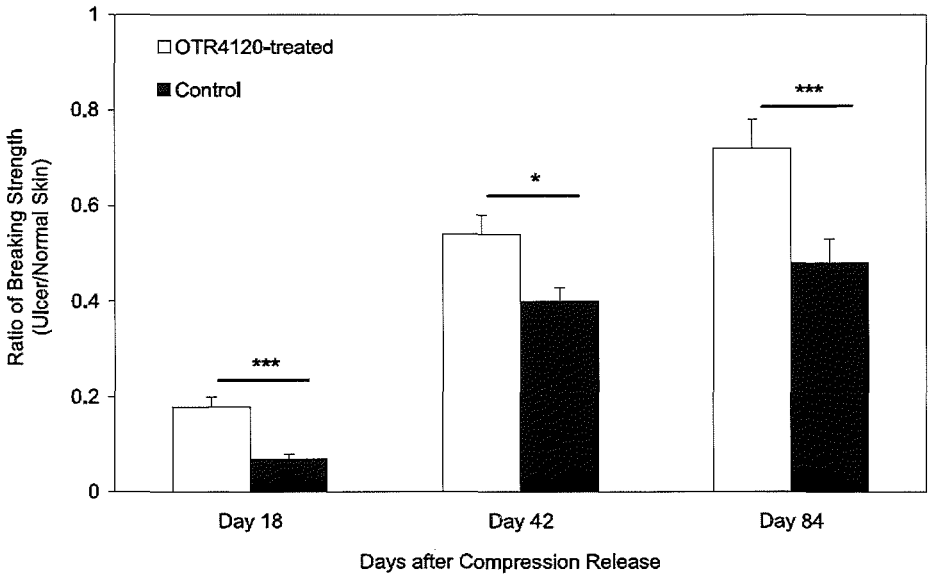

Figure 7. Ratio of the wound breaking strength of ulcer tissue compared with normal skin tissue on days 18, 42, and 84, respectively. Data are presented as means ± SEM. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ indicate significant differences between treated groups and control groups.

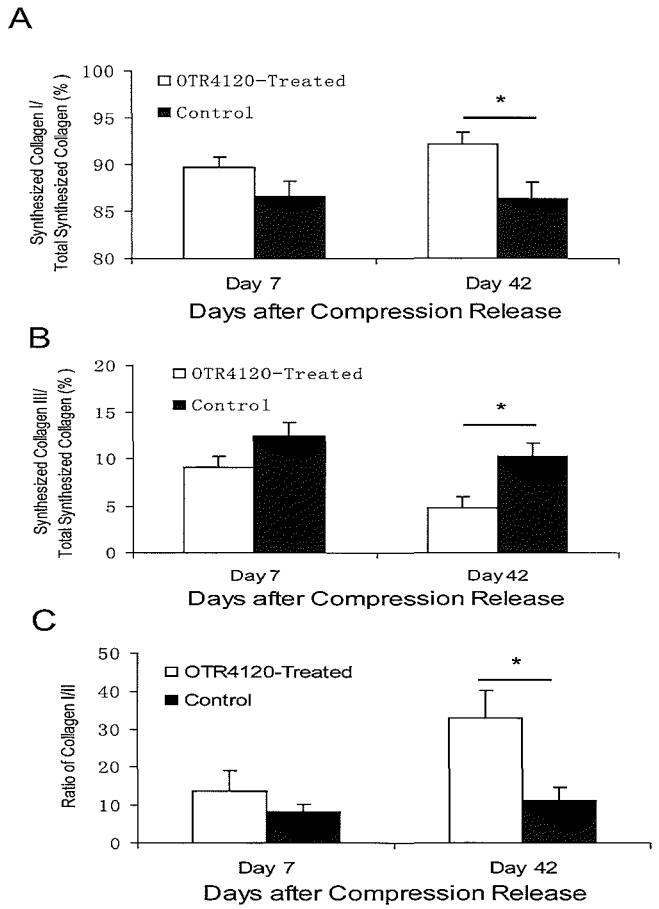

Figure 8. Collagen type I and III biosynthesis in ulcer tissues in control and OTR4120-treated rats. Tissue samples were ex vivo labelled with (^{3}H) hydroxyproline and digested by pepsin. Pepsin-soluble collagen types III and I were determined by SDS-PAGE. The percentage of synthesized collagen type I to total synthesized collagen (A), the percentage of synthesized collagen type III to total synthesized collagen (B), and the ratio of collagen type I/collagen type III (C). *$p < 0.05$ indicates significant differences between treated groups and control groups.

DISCUSSION

Matrix therapy aims to facilitate and potentiate the intrinsic tissue self-regeneration capability by restoring the natural wound microenvironment. In this study, we demonstrate that matrix therapy with OTR4120 reduces diabetic ulcer healing time, improves healing quality by reducing

inflammation, increasing nerovascularization and angiogenesis, reducing collagen type III, and enhancing the ulcer biomechanical strength restoration.

Excess inflammation, associated with a prolonged persistence of neutrophil infiltration, is a consistent feature of diabetes-impaired wound healing [6]. In another study using the same wound model in normal rats, macrophages were only detectable during the first 6 weeks after wounding [14], whereas in the present study, macrophages were detectable during the entire 12-week observation period. This is an additional sign that inflammation resolution in diabetic wound healing is impaired. This impaired resolution is stimulated after OTR4120 treatment. Possible mechanisms of the anti-inflammatory property of OTR4120 might include its effect on inhibiting plasmin and neutrophil elastase activity [19-20] and increasing the contents of TGF-β1 and VEGF that is demonstrated in this study. VEGF is known to induce macrophage apoptosis through stimulation of a tumour necrosis factor [21]. The observed increase of TGF-β1 and VEGF in wounds of OTR4120-treated animals without an OTR4120 effect on TGF-β1 and VEGF gene transcription further strengthens their sequestering/protecting/stabilizing role on GFs.

Increased amounts of collagen type III relative to collagen type I at the later phases of wound healing are often associated with excessive scarring during tissue remodeling [22]. Ex vivo radiolabeling of collagen allowed us to compare the relative rates of biosynthesis of different collagen types that are thought to reflect collagen synthesis in the wounds directly before sampling. We observed that OTR4120 treatment reduced collagen type III synthesis and increased collagen type I synthesis at the later phases of wound healing. The antifibrotic effects of OTR4120 have been reported in healing Crohn's disease intestinal biopsies [23] and burned rat skin [11]. This effect is consistent with the increased ulcer breaking strength after OTR4120 treatment. However, the underlying mechanisms of action of OTR4120 in regulating the relative proportion of collagen type I and type III during matrix remodeling remain unclear.

In addition to the anti-inflammatory and antifibrotic effects of OTR4120, this treatment also induced NO production. The diffusible, gaseous molecule NO participates in the orchestration of wound healing by modulating cytokines that are involved in wound healing [24]. NO deficiency is an important mechanism for diabetes-impaired wound healing [24-25]. Most cell types

associated with wound healing are capable of producing NO through the activity of NOS. iNOS is calcium independent and controlled by inflammatory cytokines. Once iNOS has induced the production of NO, the content of NO within tissues can increase >1,000-fold [25].

The above data showed that OTR4120 treatment revealed a positive effect on wound healing. The enhanced wound healing is often an indicator of satisfactory nutritional status and weight gain. The weight restoration was found near-significantly improved after OTR4120 treatment. This improved weight gain may contribute to the regenerating effects of OTR4120 treatment on ulcer healing.

In summary, this study shows that a synthetic HS-GAG mimetic OTR4120 accelerates and improves diabetic pressure ulcer healing in rats. It suggests that OTR4120 treatment may be a promising matrix therapy for diabetes-impaired wounds.

ACKNOWLEDGEMENTS

This research was supported by a grant from the Nuts Ohra Foundation (the Netherlands).

M.T. researched data and wrote the manuscript. B.T. researched data and edited the immunohistochemistry images. P.S., I.M.H., E.M.G.F., and M.G. researched data. S.E.R.H. contributed to the discussion. J.W.v.N. is the guarantor of this work and, as such, had full access to all the data.

Parts of this study were presented in abstract form at the 14th European Conference of Scientists and Plastic Surgeons, Pamplona, Spain, 28–29 October 2011.

The authors thank Dr. V. Barbier and Dr. P. Kern (Laboratoire de Recherches sur la Croissance Cellulaire of Université Paris XII, Créteil, France) for their help in collagen biosynthesis measurements and OTR3 (Paris, France) for providing OTR4120.

REFERENCES

1. Lioupis, C., Effects of diabetes mellitus on wound healing: an update. J Wound Care, 2005. 14(2): p. 84-6.
2. Maruyama, K., et al., Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing. Am J Pathol, 2007. 170(4): p. 1178-91.
3. Galiano, R.D., et al., Topical Vascular Endothelial Growth Factor Accelerates Diabetic Wound Healing through Increased Angiogenesis and by Mobilizing and Recruiting Bone Marrow-Derived Cells. Am J Pathol, 2004. 164(6): p. 1935-1947.
4. Lobmann, R., et al., Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. Diabetologia, 2002. 45(7): p. 1011-6.
5. Schultz, G.S. and A. Wysocki, Interactions between extracellular matrix and growth factors in wound healing. Wound Repair Regen, 2009. 17(2): p. 153-62.
6. Eming, S.A., T. Krieg, and J.M. Davidson, Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol, 2007. 127(3): p. 514-25.
7. Menke, N.B., et al., Impaired wound healing. Clin Dermatol, 2007. 25(1): p. 19-25.
8. Rouet, V., et al., A synthetic glycosaminoglycan mimetic binds vascular endothelial growth factor and modulates angiogenesis. J Biol Chem, 2005. 280(38): p. 32792-800.
9. Rouet, V., et al., Heparin-like synthetic polymers, named RGTAs, mimic biological effects of heparin in vitro. J Biomed Mater Res A, 2006. 78(4): p. 792-7.
10. Barritault, D. and J.P. Caruelle, [Regenerating agents (RGTAs): a new therapeutic approach]. Ann Pharm Fr, 2006. 64(2): p. 135-44.
11. Garcia-Filipe, S., et al., RGTA OTR4120, a heparan sulfate mimetic, is a possible long-term active agent to heal burned skin. J Biomed Mater Res A, 2007. 80(1): p. 75-84.
12. Tong, M., et al., RGTA OTR 4120, a heparan sulfate proteoglycan mimetic, increases wound breaking strength and vasodilatory capability in healing rat full-thickness excisional wounds. Wound Repair Regen, 2008. 16(2): p. 294-9.
13. Tong, M., et al., Stimulated neovascularization, inflammation resolution and collagen maturation in healing rat cutaneous wounds by a heparan sulfate glycosaminoglycan mimetic, OTR4120. Wound Repair Regen, 2009. 17(6): p. 840-52.
14. Tong, M., et al., Heparan sulfate glycosaminoglycan mimetic improves pressure ulcer healing in a rat model of cutaneous ischemia-reperfusion injury. Wound Repair Regen, 2011. 19(4): p. 505-14.
15. Zuijdendorp, H.M., et al., Significant reduction in neural adhesions after administration of the regenerating agent OTR4120, a synthetic glycosaminoglycan mimetic, after peripheral nerve injury in rats. J Neurosurg, 2008. 109(5): p. 967-73.

16. Lardenoye, J.W., J.A. Thiefaine, and P.J. Breslau, Assessment of incidence, cause, and consequences of pressure ulcers to evaluate quality of provided care. Dermatol Surg, 2009. 35(11): p. 1797-803.
17. Stark, J.M., et al., Detection of enhanced neutrophil adhesion to parainfluenza-infected airway epithelial cells using a modified myeloperoxidase assay in a microtiter format. J Virol Methods, 1992. 40(2): p. 225-42.
18. Woessner, J.F., Jr., The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys, 1961. 93: p. 440-7.
19. Ledoux, D., et al., Human plasmin enzymatic activity is inhibited by chemically modified dextrans. J Biol Chem, 2000. 275(38): p. 29383-90.
20. Meddahi, A., et al., FGF protection and inhibition of human neutrophil elastase by carboxymethyl benzylamide sulfonate dextran derivatives. Int J Biol Macromol, 1996. 18(1-2): p. 141-5.
21. Melissa L. Petreaca, M.Y., Carl Ware, Manuela M. Martins-Green,, Vascular endothelial growth factor promotes macrophage apoptosis through stimulation of tumor necrosis factor superfamily member 14 (TNFSF14/LIGHT). Wound Repair and Regeneration, 2008. 16(5): p. 602-614.
22. Widgerow, A.D., Cellular/extracellular matrix cross-talk in scar evolution and control. Wound Repair Regen, 2011. 19(2): p. 117-33.
23. Alexakis, C., et al., Reversal of abnormal collagen production in Crohn's disease intestinal biopsies treated with regenerating agents. Gut, 2004. 53(1): p. 85-90.
24. Schwentker, A., et al., Nitric oxide and wound repair: role of cytokines? Nitric Oxide, 2002. 7(1): p. 1-10.
25. Boykin, J.V., Jr., Wound nitric oxide bioactivity: a promising diagnostic indicator for diabetic foot ulcer management. J Wound Ostomy Continence Nurs. 37(1): p. 25-32; quiz 33-4.

CHAPTER 6

General Discussion

REGULATION OF THE FUNCTION OF THE ECM IN WOUND TREATMENT

Wounds, especially therapy-refractory chronic wounds, are a significant health care concern. To treat patients with chronic wounds is difficult due to the dynamics and complexity of the healing system, in which many interdependences and redundancies are involved. To manage such a complex system, an important way is to create an appropriate environment where the system can unfold the self-healing and self-control mechanism of natural wound healing.

This concept is comparable to seeding the seeds in the soil. Many treatments often include the external interventions to bring growth factors, (stem) cells, matrix proteins, or engineered skin substitutes (i.e. seeds) to wound ECM (i.e. soil). However, the key issue is how to keep these "seeds" alive and make them integrated into soil. Like an appropriate soil is essential for seeding, the functional ECM is prerequisite for the successful wound healing. However, the ECM and the function of the ECM are often disturbed in chronic wound healing.

Regulation of the function of the ECM in wound tissue is complex and critically dependent on the interactions of the ECM with cells, growth factors, cytokines, and many biological molecules. In wound healing, large amounts of active cytokines and growth factors are required; therefore, the modifications in the availability and activity of growth factors and cytokines are essential. These modifications largely rely on the interactions between the ECM and growth factors [1].

A heparan sulfate glycosaminoglycan (HS-GAG) mimetic, OTR4120, can restore or improve the interactions between the ECM and growth factors through repositioning growth factors back to the ECM, protecting them from degradation, activating their receptors, and sequestering them as required. In this way, OTR4120 regulates and improves the function of the ECM. The functioning ECM in wound tissue environment allows tissue to unfold its self-healing capability (i.e. natural wound healing mechanisms) that may shift wound repair to wound regeneration. This is the rationale behind the strategy to use matrix therapy with OTR4120 in wound treatment (Chapter 1).

OTR4120 MATRIX THERAPY IN ACUTE WOUND HEALING

Most acute cutaneous wounds heal rapidly. However, the outcome may be accompanied by mechanical weakness, functional loss, and cosmetic changes [2]. An ultimate reason for these imperfect outcomes is that most wounds heal by repair, not by regeneration. OTR4120, which is hypothesized to act as a tissue-regenerating agent, may improve the outcome of wound healing.

In this thesis, a surgical full-thickness excisional wound model was selected to study the effects of OTR4120 on acute wound healing (Chapter 2 and 3). Surgical wounds are the most common acute wounds in clinics. Acute healing of full-thickness wounds clearly demonstrates the three-phase wound healing process. In this model, removal of full-thickness skin tissue requires filling the gap by granulation tissue formation, reepithelialization, vascularization, and collagen deposition. Finally these wounds develop into the functional equivalent of normal skin. Besides, an excisional wound allows a relatively large wounded area for sampling and performing measures. Based on above consideration, we chose the full-thickness excisional wound model as a platform to evaluate the therapeutic efficacy of OTR4120 on healing acute wounds.

Wound breaking strength, microcirculation, and wound closure rate were first evaluated in this acute wound model. Matrix therapy with OTR4120 protects growth factors in the ECM in wounds, which facilitates and regulates the natural healing process. OTR4120 therefore may not accelerate the speed of healing in a specific phase, but will improve the overall quality of repair. Therefore, we first verified the therapeutic efficacy of OTR4120 by evaluating the endpoint parameters of tissue repair, such as wound breaking strength, microcirculation and wound closure rate.

Breaking strength restoration finalizes in the tissue remodeling phase. Also it is an accumulated outcome contributed by many events from the up-stream phases of wound healing [3-4]. Therefore, the restoration of biomechanical strength is a powerful parameter for evaluating the endpoint product of wound healing (Chapter 2).

Microcirculation also is an important parameter in assessing wound healing potential. In this surgical model, the re-established blood flow and vascular response to the local heat provocation (i.e. vasodilatory capability) was evaluated. Vasodilatory capability is not only related to the number of capillaries but also to the endothelial function (Chapter 2).

Our work showed that OTR4120 treatment had a long-term effect on increasing wound breaking strength and vasodilatory capability in this acute wound-healing model. The wound closure rate was not found influenced by OTR4120 treatment (Chapter 2). This may be because the rapid wound contraction of rodent wounds makes the wound closure measurement imprecise and fallible [5]. However, we did observe that OTR4120 accelerates re-epithelialization in this surgical wound model (Chapter 3). Increased re-epithelialization has a high prediction of beneficial effect of OTR4120 also on wound closure time.

We also investigated the effects of OTR4120 treatment on different wound healing phases and found that OTR4120 had beneficial effects on inflammation resolution, epidermal proliferation, granulation tissue formation, re-epithelialization, neovascularization, and collagen maturation in acute wound healing (Chapter 3). The underlying mechanisms of action of OTR4120 on improving acute wound healing may be due to the property of OTR4120 in protecting growth factors and cytokines. As was noted in the introduction, the expression of growth factors and cytokines regulates the feedforward/feedback loops and ensures an orderly, coordinated, and self-limiting progression of acute wound healing [6].

These beneficial effects of OTR4120 on acute wound healing prompted us to further verify its effects on impaired wound healing.

IMPAIRED WOUND HEALING ANIMAL MODELS

The necessity and limitation of impaired wound models

Although a chronic wound is impossible to be exactly mimicked in an animal model, impaired wound healing models that replicate human chronic wounds on the certain aspects are needed in

wound research for ensuring an in-depth study of biological mechanisms and for testing the efficacy of therapeutic agents or interventions [7-8].

It has to be noted that the etiology of human chronic wounds is different from that which can be mimicked in animals. Human chronic wounds are often created following a combination of impaired circulation, poor nutrition, restricted physical activity, and comorbid physiological imbalance. Such complexity is difficult to be replicated in an animal model [9-10]. In addition, there are numerous anatomical and physiological differences between human and animal [9]. Therefore, a wound model exactly comparable with chronic human wounds is nonexistent [8]. However, human chronic wounds can be simplified and mimicked in animal models by focusing on the isolated segments of human chronic wound problems [9-11]. In this thesis, a chronic wound related systemic factor (i.e. diabetes) and/or a chronic wound related local factor (i.e. pressure/ischemia) were focused in two ulcer models

These two ulcer models include an I-R injury-induced pressure ulcer model (Chapter 4) and an experimental diabetic I-R injury-induced pressure ulcer model (Chapter 5). They were used to represent experimental impaired wound healing. The impairment and the clinical relevance of these two ulcer models are discussed below:

Impairment of these two ulcer models

These two ulcer models, which were created in normal rats and STZ-induced diabetic rats, were both proven delayed in the time of complete wound healing compared with the surgical wound model. The complete ulcer closure of the pressure ulcer model in normal rats was found to be 2 weeks delayed, and the complete ulcer closure in the diabetic ulcer model was found to be 4 weeks delayed compared with the surgical wound model. Moreover, both ulcer models displayed persistent neutrophil and macrophage infiltration: for the diabetic ulcer model, the macrophages were persistently present throughout the entire observation period (i.e. 12 weeks after the compression release) (Chapter 5), and for the ulcer model in normal rats, the macrophage infiltration were persistently present within 6 weeks after the compression release (Chapter 4), whereas, the surgical wound model displayed the neutrophil and macrophage

infiltration only in the first 3 weeks after wounding. This indicates the impairment of inflammation resolution of those ulcer models.

Clinical relevance

Pressure ulcers are one of the most common chronic wound types. Ischemia-reperfusion (I-R) injury is a significant factor of the etiology of ulceration [12]. Pressure exceeding tissue perfusion results in a period of ischemia that may be followed by reperfusion. This situation was mimicked in the pressure ulcer model conducted in this thesis. Consistent with clinical ulceration, similar clinical symptoms, such as edema, erythema, discoloration and final necrosis, were also observed in this pressure ulcer model (Chapter 4).

Diabetes is a common, worldwide disease. The world prevalence of diabetes is 6.4% of the population in the year 2010 and is expected to increase to 7.7% by the year 2030 [13]. We combined pressure ulcers with diabetes to create another impaired wound-healing model: the diabetic pressure ulcer model (Chapter 5).

OTR4120 MATRIX THERAPY IN IMPAIRED WOUND HEALING

Impaired wound healing is characterized by excessive inflammation, impaired angiogenesis, abnormality of growth factor, and elevated ECM degradation [14-15]. Matrix therapy that reconstitutes/incorporates ECM and growth factors and thus restores natural cell-ECM and cell-cell signaling may be beneficial for improving impaired wound healing.

Successful tissue repair requires both inflammation and inflammation resolution [6, 16-17]. In this thesis, evidence of an impaired inflammation resolution was found in those two impaired wound models. In the diabetic ulcer model, monocytes/macrophages were persistently present throughout the entire 12-week observation period (Chapter 5). And in the ulcer model in normal rats, monocytes/macrophages were also detected within 6 weeks after ulceration (Chapter 4). In contrast, in the surgical wound model, monocytes/macrophages were nonexistent in 3 weeks after wounding and the inflammation peak was 1 week earlier than that in the normal ulcer model (Chapter 3). A major consequence of the persistent inflammation cascade is an excessive

amount of MMPs that overwhelms TIMPs thus contributes to the unbalanced proteolytic activities in the chronic wound environment. This highly proteolytic environment degrades the ECM and growth factors and also may lead to the unsatisfactory results of many current treatments [18].

In this thesis, the anti-inflammation property of OTR4120 was found on both acute and impaired wound healing. The mechanism that limits or down-regulates inflammation includes: (1) up regulation of anti-inflammatory cytokines such as TGF-β_1 and IL-10 [19-20]; (2) inhibition of proteinases; (3) down-regulation of proinflammatory cytokines such as IL-1, IL-6, and tumour necrosis factor-α (TNF-α); (4) up-regulation of apoptosis; and (5) reconstitution of the microvascular permeability, which contributes to the cessation of local chemoattractants, synthesis of anti-inflammatory mediators, apoptosis, and lymphatic drainage [21]. Based on the results obtained in this thesis, the anti-inflammation effect of OTR4120 is likely conducted through increasing the number of anti-inflammatory cytokines (i.e. TGF-β_1) in the wound (Chapter 4 and 5), and/or increasing microvascular permeability by the increased local content of VEGF (Chapter 3, 4, and 5). VEGF, the most potent direct-acting-angiogenic protein, is a diffusible endothelial cell-specific mitogen and angiogenic factor. It can increase vascular permeability [22-24]. Taken together, OTR4120 improves bioavailability of growth factors and improves the natural wound microenvironment. The optimal wound environment will disrupt the inflammatory cycle and allow full induction of anti–inflammation pathways. However, the precise mechanism of the anti-inflammation effect of OTR4120 is in need of further investigation.

Angiogenesis is essential for wound healing. Insufficient angiogenesis is a hallmark feature of chronic wounds. Therapy for chronic wounds often incorporates a number of angiogenesis-stimulating strategies such as growth factor therapy [23, 25-26]. However, there are some concerns regarding to the safety and the efficacy of this therapy. One is the short circulating half-life of recombinant growth factors. Another is the reduced bioavailability of growth factors in a highly proteolytic chronic wound environment [27]. These fast metabolic and proteolytic degradations make it hard to create a local effective concentration of growth factors.

One possible solution to increase the local bioavailability of growth factors is to use an appropriate delivery system. Gene transfer is a potential modality to increase quantity of growth factors in the wound, but its safety has to be proven before it can be applied in clinics [28-30]. In this thesis, OTR4120 was found to increase the local content of growth factors (i.e. VEGF, PDGF-A, and TGF-β_1) at protein levels by protein stabilization but not at mRNA levels. This result proves the growth factors protection property of OTR4120. This property implies that OTR4120 may be an alternative of growth factor gene therapy to increase the local bioavailability of growth factors during wound treatment (Chapter 5).

Scar formation is the physiological end point of adult cutaneous wound repair. However, excessive scarring such as hypertrophic scars and keloids may cause significant health problems and their treatment remains undefined [14, 31-34]. Although the precise mechanism of scar formation is not well understood, there is substantial evidence that inflammation, reepithelialization, and the delicate balance between ECM degradation and synthesis plays an important role in the pathophysiolofy of scar formation [2, 14, 34]. In this thesis, OTR4120 was found to increase the total collagen content and wound breaking strength (Chapter 4 and 5). Furthermore, it showed that in the later stages of wound healing, OTR4120 reduced collagen type III synthesis and increased collagen type I synthesis (Chapter 5). Increased amounts of collagen type III relative to collagen type I at the later stages of wound healing are often associated with excessive scarring (e.g. keloids) [35]. The similar anti-fibrotic effects of OTR4120 have been reported in healing Crohn's disease [36] and rat burns [37]. In the surgical wound model in this thesis, OTR4120 was found to promote reepithelialization and collagen maturation (Chapter 3). In both acute and impaired wound models presented in this thesis, OTR4120 was observed to reduce inflammation, especially to stimulate the inflammation resolution, and to consistently enhance wound breaking strength (Chapter 3, 4, and 5). All these findings suggest that OTR4120 has a potential in mitigating scar formation.

CONCLUSION

The aim of this thesis was to verify the effects of OTR4120 on wound healing and unravel the underlying mechanisms and the implications of OTR4120 in clinical application. The work

presented in this thesis demonstrates that OTR4120 matrix therapy has positive effects on improving both acute and impaired wound healing. These benefits for wound healing are associated with the stimulated inflammation resolution, induced nerovascularization, increased the content of growth factors (i.e. VEGF, TGF-β_1, and PDGF), enhanced NO production, increased collagen bio-synthesis, and promoted collagen maturation and regulation. These findings provide a rationale for the clinical application of OTR4120 for the treatment of cutaneous wounds.

FUTURE PERSPECTIVES

Following the work presented in this thesis, further studies unveiling the detained mechanisms of action of OTR4120 in wound healing and its comprehensive efficacy as a regenerating agent for wound treatment are required. The possible directions of future research may include:

Further study the detailed mechanisms of action of OTR4120

One limitation of this thesis is that the mechanistic studies of action of OTR4120 at cellular and molecular levels are inadequate. This limitation is in part due to a lack of experimental tools (i.e. antibody against OTR4120 or labelled OTR4120) to track the location and behaviour of OTR4120 after its application in animal models. In addition, in order to verify both reliability and validity of the effects of OTR4120 in wound healing, in this thesis, the similar outcome measures that involve the three phases of healing process were evaluated in those three wound models. This experimental design yielded the robust and comparable results, however, the detailed mechanisms of action of OTR4120 in a given healing phase or in a specific wound model were not intensively studied. However, the observed overall picture of the effects of OTR4120 on wound healing directs the future detailed mechanistic studies of action of OTR4120. The focus could be on inflammation and the balance between ECM synthesis and turnover.

The excessive inflammation in chronic wounds is associated with many factors. In this thesis, PMN infiltration and monocyte/ macrophage accumulation were mainly evaluated. Future studies could explore the potential of OTR4120 on targeting the increased proinflammatory cytokines (i.e. IL-1 and TNF-α), matrix proteolytic activities (i.e. neutrophil elastase, MMP-8, and gelatinase), and reactive oxygen species (i.e. H_2O_2, O_2^-).

In this thesis the effect of OTR4120 on tissue remodeling was mainly studied on collagen synthesis and maturation. The potential of OTR4120 on characterizing the altered ECM turnover in chronic wounds could be an interesting/important direction of future studies [38].

Future research on the potential of OTR4120 on preventing ulceration

The prevention of ulceration and the treatment of ulcer on the early stages of healing is a primary goal of ulcer management. Although the experiments presented in this thesis were not originally designed for the evaluation of ulcer prevention, OTR4120 was applied within the ulceration period, and found to reduce the ulcer grade (i.e. degree of damage to the tissue) (Chapter 4 and 5). This suggests that OTR4120 has a potential in preventing pressure ulceration. Future experiments, specifically designed for ulcer prevention, may substantiate this potential of OTR4120 in ulcer prevention.

Future research on the potential of OTR4120 on reducing scar formation

Excessive scarring in the form of keloids and hypertrophic scars continues to be a clinical problem [39]. OTR4120 was observed to reduce the biosynthesis of collagen type III (Chapter 5), accelerate re-epithelialization (Chapter 3), stimulate inflammation resolution, and enhance wound breaking strength (Chapter 3, 4, and 5). These results showed the ability of OTR4120 to improve the quality of wound healing, which may be associated to its potential on reducing scar formation. Next step, by using a scarring model (i.e. a rabbit ear model, immunodeficient animal model, or porcine model) [39-40], the anti-scarring effect of OTR4120 could be verified. The pathogenesis of scarring is complex and much of it remains unexplored [41-42]. The future studies could evaluate the altered scarring-related gene expressions [43-45], gene products (i.e. TGF-β1, FGFs, IL-6, IL-8, and lysyl oxidase) [46-48], and collagen regulation and maturation.

Future research on the efficacy of combination of OTR4120 and growth factors on wound healing

The clinical success of the current exogenous growth factor therapy is limited due to the sequential availability and function of growth factors in healing process (i.e. individual growth factor targets its specific-involved healing process temporally and spatially) [49-50] and the highly proteolytic environment of chronic wounds that could degrade exogenously applied growth factors [27, 51]. In addition, the difficulty in finding optimal dosage and timing of growth factor application also limits the efficacy of this therapy. Importantly, because the extensive distribution and the multiple function of growth factors, their overdoses will increase the risk of potential side effects such as tumor induction, abnormal cell transformation, and hypertrophic scar formation [52-53]. Combining OTR4120 with growth factors may minimize these limitations, because OTR4120 acts as a reservoir for the exogenously applied growth factors and allows growth factors to be sequestered temporally and spatially as needed in the different wound healing phases.

On the standpoint of OTR4120, the exogenously applied growth factors, in turn, ensure the efficacy of OTR4120 treatment, as theoretically OTR4120 matrix therapy is only effective if the endogenous growth factors exist or can be produced in wounds. Moreover, through combining with exogenous growth factors, OTR4120 will have an enriched reservoir of growth factors that ensures the growth factor-mediated activation of cellular functions that is essential for wound healing and also enables the full communication of cell-cell and cell-ECM during wound healing.

Overall, the complementary and synergistic effects of combination of OTR4120 and growth factors may implicate a promising therapeutic approach for wound treatment.

REFERENCES

1. Schultz, G.S. and A. Wysocki, Interactions between extracellular matrix and growth factors in wound healing. Wound Repair Regen, 2009. 17(2): p. 153-62.
2. Robson, M.C., D.L. Steed, and M.G. Franz, Wound healing: biologic features and approaches to maximize healing trajectories. Curr Probl Surg, 2001. 38(2): p. 72-140.
3. Chodorowska, G. and D. Rogus-Skorupska, Cutaneous wound healing. Ann Univ Mariae Curie Sklodowska Med, 2004. 59(2): p. 403-7.
4. Clark, R.A., Biology of dermal wound repair. Dermatol Clin, 1993. 11(4): p. 647-66.
5. Galiano, R.D., et al., Quantitative and reproducible murine model of excisional wound healing. Wound Repair Regen, 2004. 12(4): p. 485-92.
6. Menke, N.B., et al., Impaired wound healing. Clin Dermatol, 2007. 25(1): p. 19-25.
7. Lindblad, W.J., Considerations for selecting the correct animal model for dermal wound-healing studies. J Biomater Sci Polym Ed, 2008. 19(8): p. 1087-96.
8. Gottrup, F., M.S. Agren, and T. Karlsmark, Models for use in wound healing research: A survey focusing on in vitro and in vivo adult soft tissue. Wound Repair and Regeneration, 2000. 8(2): p. 83-96.
9. Dorsett-Martin, W.A., Rat models of skin wound healing: a review. Wound Repair Regen, 2004. 12(6): p. 591-9.
10. Gottrup, F., M.S. Agren, and T. Karlsmark, Models for use in wound healing research: a survey focusing on in vitro and in vivo adult soft tissue. Wound Repair Regen, 2000. 8(2): p. 83-96.
11. Davidson, J.M., Animal models for wound repair. Arch Dermatol Res, 1998. 290 Suppl: p. S1-11.
12. Jiang, L.P., et al., Ischemia-reperfusion injury-induced histological changes affecting early stage pressure ulcer development in a rat model. Ostomy Wound Manage, 2011. 57(2): p. 55-60.
13. Shaw, J.E., R.A. Sicree, and P.Z. Zimmet, Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract, 2010. 87(1): p. 4-14.
14. Eming, S.A., T. Krieg, and J.M. Davidson, Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol, 2007. 127(3): p. 514-25.
15. Medina, A., et al., Pathophysiology of chronic nonhealing wounds. J Burn Care Rehabil, 2005. 26(4): p. 306-19.

16. Martin, P. and S.J. Leibovich, Inflammatory cells during wound repair: the good, the bad and the ugly. Trends in Cell Biology, 2005. 15(11): p. 599-607.
17. Nathan, C., Points of control in inflammation. Nature, 2002. 420(6917): p. 846-52.
18. Lauer, G., et al., Generation of a novel proteolysis resistant vascular endothelial growth factor165 variant by a site-directed mutation at the plasmin sensitive cleavage site. FEBS Lett, 2002. 531(2): p. 309-13.
19. Werner, F., et al., Transforming growth factor-beta 1 inhibition of macrophage activation is mediated via Smad3. J Biol Chem, 2000. 275(47): p. 36653-8.
20. Sato, Y., T. Ohshima, and T. Kondo, Regulatory role of endogenous interleukin-10 in cutaneous inflammatory response of murine wound healing. Biochem Biophys Res Commun, 1999. 265(1): p. 194-9.
21. Eming, S.A., T. Krieg, and J.M. Davidson, Inflammation in Wound Repair: Molecular and Cellular Mechanisms. 0000. 127(3): p. 514-525.
22. Dvorak, H.F., et al., Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol, 1995. 146(5): p. 1029-39.
23. Werner, S. and R. Grose, Regulation of wound healing by growth factors and cytokines. Physiol Rev, 2003. 83(3): p. 835-70.
24. Ferrara, N. and T. Davis-Smyth, The biology of vascular endothelial growth factor. Endocr Rev, 1997. 18(1): p. 4-25.
25. Bennett, S.P., et al., Growth factors in the treatment of diabetic foot ulcers. Br J Surg, 2003. 90(2): p. 133-46.
26. Fu, X., et al., Engineered growth factors and cutaneous wound healing: success and possible questions in the past 10 years. Wound Repair Regen, 2005. 13(2): p. 122-30.
27. Chen, S.M., et al., Ability of chronic wound fluids to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of proteinase inhibitors. Wound Repair Regen, 1997. 5(1): p. 23-32.
28. Isner, J.M., et al., Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet, 1996. 348(9024): p. 370-4.
29. Galeano, M., et al., Effect of recombinant adeno-associated virus vector-mediated vascular endothelial growth factor gene transfer on wound healing after burn injury. Crit Care Med, 2003. 31(4): p. 1017-25.
30. Deodato, B., et al., Recombinant AAV vector encoding human VEGF165 enhances wound healing. Gene Ther, 2002. 9(12): p. 777-85.
31. Bayat, A., D.A. McGrouther, and M.W. Ferguson, Skin scarring. Bmj, 2003. 326(7380): p. 88-92.

32. Haverstock, B.D., Hypertrophic scars and keloids. Clin Podiatr Med Surg, 2001. 18(1): p. 147-59.
33. Slemp, A.E. and R.E. Kirschner, Keloids and scars: a review of keloids and scars, their pathogenesis, risk factors, and management. Curr Opin Pediatr, 2006. 18(4): p. 396-402.
34. Jinnin, M., Mechanisms of skin fibrosis in systemic sclerosis. J Dermatol, 2010. 37(1): p. 11-25.
35. Widgerow, A.D., Cellular/extracellular matrix cross-talk in scar evolution and control. Wound Repair Regen, 2011. 19(2): p. 117-33.
36. Alexakis, C., et al., Reversal of abnormal collagen production in Crohn's disease intestinal biopsies treated with regenerating agents. Gut, 2004. 53(1): p. 85-90.
37. Garcia-Filipe, S., et al., RGTA OTR4120, a heparan sulfate mimetic, is a possible long-term active agent to heal burned skin. J Biomed Mater Res A, 2007. 80(1): p. 75-84.
38. Blakytny, R. and E. Jude, The molecular biology of chronic wounds and delayed healing in diabetes. Diabet Med, 2006. 23(6): p. 594-608.
39. Morris, D.E., et al., Acute and chronic animal models for excessive dermal scarring: quantitative studies. Plast Reconstr Surg, 1997. 100(3): p. 674-81.
40. Ramos, M.L., A. Gragnani, and L.M. Ferreira, Is there an ideal animal model to study hypertrophic scarring? J Burn Care Res, 2008. 29(2): p. 363-8.
41. Satish, L. and S. Kathju, Cellular and Molecular Characteristics of Scarless versus Fibrotic Wound Healing. Dermatol Res Pract, 2010. 2010: p. 790234.
42. Shih, B. and A. Bayat, Genetics of keloid scarring. Arch Dermatol Res, 2010. 302(5): p. 319-39.
43. Wu, J., et al., Gene expression of early hypertrophic scar tissue screened by means of cDNA microarrays. J Trauma, 2004. 57(6): p. 1276-86.
44. Naitoh, M., et al., Gene expression in human keloids is altered from dermal to chondrocytic and osteogenic lineage. Genes Cells, 2005. 10(11): p. 1081-91.
45. Satish, L., et al., Gene expression patterns in isolated keloid fibroblasts. Wound Repair Regen, 2006. 14(4): p. 463-70.
46. Verrecchia, F. and A. Mauviel, Transforming growth factor-beta and fibrosis. World J Gastroenterol, 2007. 13(22): p. 3056-62.
47. Liechty, K.W., N.S. Adzick, and T.M. Crombleholme, Diminished interleukin 6 (IL-6) production during scarless human fetal wound repair. Cytokine, 2000. 12(6): p. 671-6.
48. Colwell, A.S., et al., Early-gestation fetal scarless wounds have less lysyl oxidase expression. Plast Reconstr Surg, 2006. 118(5): p. 1125-9; discussion 1130-1.

49. Krishnamoorthy, L., H.L. Morris, and K.G. Harding, Specific growth factors and the healing of chronic wounds. J Wound Care, 2001. 10(5): p. 173-8.
50. Robson, M.C., T.A. Mustoe, and T.K. Hunt, The future of recombinant growth factors in wound healing. Am J Surg, 1998. 176(2A Suppl): p. 80S-82S.
51. Yager, D.R. and B.C. Nwomeh, The proteolytic environment of chronic wounds. Wound Repair Regen, 1999. 7(6): p. 433-41.
52. Favoni, R.E. and A. de Cupis, The role of polypeptide growth factors in human carcinomas: new targets for a novel pharmacological approach. Pharmacol Rev, 2000. 52(2): p. 179-206.
53. Russell, P.J., S. Bennett, and P. Stricker, Growth factor involvement in progression of prostate cancer. Clin Chem, 1998. 44(4): p. 705-23.

CHAPTER 7

Appendices

SUMMARY

Wounds, especially chronic wounds, create a significant burden on both the patients and the healthcare resources. Improving wound healing is therefore of paramount importance and intensively studied.

Heparan sulfate glycosaminoglycan (HS-GAG) mimetic OTR4120 replaces the destroyed HS-GAG in the extracellular matrix (ECM) of wounds. It repositions heparin-binding growth factors back into the ECM, protects growth factors from degradation and improves their bioavaliability. In this way, OTR4120 restores the interactions between the ECM and growth factors and thus improves the function of the ECM. The improved functional ECM in wound environment unfolds natural wound healing mechanisms (i.e. self-regeneration capability) and likely triggers tissue regeneration (**Chapter 1**).

In this thesis, the efficacy of a matrix therapy with OTR4120 was verified on both acute and impaired cutaneous wound healing models. The underlying mechanisms of action of OTR4120 treatment were also partly unraveled.

In Chapter 2, the effect of OTR4120 treatment was first verified on an acute (i.e. a surgical full-thickness excisional) wound model. Two important clinical-relevant outcomes of wound healing: wound breaking strength and vasodilatory capability, were found to be improved following OTR4120 treatment.

In Chapter 3, we further investigated the mechanisms of action of OTR4120 treatment on improving acute wound healing. OTR4120 treatment was found to stimulate inflammation resolution, increase neovascularization and promote epidermal migration and proliferation during reepithelialization. In addition, the granulation tissue formation and collagen maturation were improved in OTR4120-treated wounds. These beneficial effects of OTR4120 on acute wound healing prompted us to further investigate its effects on impaired wound healing.

In Chapter 4, a pressure ulcer model, induced by ischemia-reperfusion (I-R) injury was utilized to verify the effect of OTR4120 on impaired wound healing. This model created sustained skin incomplete necrosis. The results showed that OTR4120 treatment decreased the area of incomplete necrosis. And also the inflammatory response was reduced. Angiogenesis and the contents of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor beta-1 (TGF-β_1) were increased in OTR4120-treated ulcers. In addition, OTR4120 treatment promoted early expression of alpha-smooth muscle actin (α-SMA) and increased collagen biosynthesis. Similar to the result of wound breaking strength restoration in acute wounds, a long-term effect of OTR4120 treatment on increasing ulcer breaking strength was found in this pressure ulcer model

In Chapter 5, we created this I-R injury induced pressure ulcers on streptozotocin-induced diabetic rats to verify the effect of OTR4120 on experimental diabetes impaired wound healing. OTR4120 treatment was found to significantly reduce the degree of ulceration and accelerate the ulcer healing. The underlying mechanism was associated with the reduced neutrophil infiltration and macrophage accumulation as well as the enhanced angiogenesis. In addition of collagen content measured by quantification of hydroxyproline, the ex vivo biosynthesis of collagen type I and type III was measured by radiolabeling of collagen during incubation of wound tissue biopsy. OTR4120 treatment was found to increase the collagen content with an increase of collagen type I biosynthesis and reduction of collagen type III biosynthesis. Similarly, restoration of the ulcer breaking strength was significantly enhanced following OTR4120 treatment.

In Chapter 6, we stated the main findings in this thesis and the author's interpretations and thoughts. This chapter also drew out the implications of OTR4120 treatment for clinical application. At the end, the possibilities for future research were elaborated.

The main findings of this thesis were that OTR4120 treatment improved both acute and impaired wound healing through resolving inflammation, stimulating angiogenesis and neovascularization, and increasing collagen synthesis, regulation, and maturation. These findings suggest that OTR4120 treatment has a therapeutic potential in treating both acute and impaired wounds.

SAMENVATTING

Wonden, in het bijzonder chronische wonden, veroorzaken een grote belasting voor zowel de patiënt (pijn, sociaal isolement) als de maatschappij (kosten van zorg, arbeidsverlies). Verbetering van de wondbehandeling is daarom van groot belang.

Heparan sulfaat glycosaminoglycaan (HS-GAG)-analoog OTR4120 kan afgebroken HS-GAG in het wondgebied vervangen. Hierdoor kunnen heparine bindende groeifactoren worden teruggeplaatst in de extracellulaire matrix (ECM) en worden ze beschermd tegen degradatie. Op deze wijze herstelt OTR4120 de natuurlijke communicatie tussen cellen, ECM en groeifactoren. Functioneel ECM in een wondgebied herstelt de natuurlijke wondgenezingsmechanismen, hetgeen kan leiden tot weefselregeneratie (Hoofdstuk 1).

In dit proefschrift wordt het effect van een matrix behandeling met OTR4120 bepaald op de genezing van normaal en vertraagd genezende wonden. Het onderliggende werkingsmechanisme van de OTR4120 behandeling wordt deels ontrafeld.

In Hoofdstuk 2 wordt het effect bepaald van de OTR4120 behandeling in een acuut 'volledige-dikte' chirurgisch wondmodel in proefdieren. Twee belangrijke klinisch relevante uitkomstmaten nemen toe als gevolg van de OTR4120 behandeling: de wondbreeksterkte en de bloedvatcapaciteit.

In Hoofdstuk 3 wordt het werkingsmechanisme van de OTR4120 behandeling op het verbeteren van de acute wondgenezing onderzocht. OTR4120 behandeling heeft een positief effect op het uitdoven van de ontstekingsreactie, verhoogt de bloedvatnieuwvorming (angiogenese) en bevordert de epidermale migratie en proliferatie tijdens de re-epithelialisatie fase. Bovendien is de granulatieweefselvorming en collageen rijping (maturatie) verbeterd in OTR4120 behandelde wonden. Deze gunstige effecten van de OTR4120 behandeling op de acute wondgenezing vormden de aanleiding om de effecten ook te onderzoeken bij een vertraagde wondgenezing.

In Hoofdstuk 4 gebruiken we een decubitus (doorligwond) diermodel dat, door een ischemie-reperfusie (IR) letsel, huidnecrose veroorzaakt. We tonen aan dat de omvang van het 141
necrosegebied is afgenomen in de OTR4120 behandelde groep. Ook de ontstekingsreactie is hier verminderd. Angiogenese en de mate van aanwezigheid van een aantal belangrijke groeifactoren (de vasculaire endotheliale groeifactor VEGF, de bloedplaatjes groeifactor PDGF, en de transformerende groeifactor TGF-ß1) is verhoogd in OTR4120 behandelde wonden. Daarnaast bevordert de OTR4120 behandeling de collageen biosynthese. Vergelijkbaar met het resultaat van de wond breeksterkte analyse in acute wonden, is ook in dit decubitus model een lange-termijn effect van de OTR4120 behandeling het verbeteren van de wondbreeksterkte.

In Hoofdstuk 5 hebben we een vertraagd genezend wondmodel vervaardigd: IR letsel in streptozotocine-geïnduceerde diabetische ratten. OTR4120 behandeling reduceert de wondgrootte en bespoedigt de genezingstijd ten opzichte van controle dieren. Een verminderde neutrofiel- en macrofaaginfiltratie en sterk verbeterde angiogenese speelt hierbij zeker een rol. Tevens zien we in de OTR4120 behandelde groep een toename van het collageengehalte, de collageen type I biosynthese alsmede een vermindering van de met littekenweefsel geassocieerde collageen type III biosynthese. Ook hier is wondbreeksterkte significant verbeterd na OTR4120 behandeling.

Hoofdstuk 6 geeft de belangrijkste bevindingen van de studies en de interpretaties en gedachten van de auteur weer. In dit hoofdstuk wordt ook de aandacht gevestigd op de implicaties van de OTR4120 behandeling ten aanzien van de klinische toepassing. Ook worden de beperkingen van dit onderzoek en de mogelijkheden voor verder onderzoek, geschetst.

De belangrijkste bevindingen van dit proefschrift zijn dat de OTR4120 behandeling zowel de acute alsmede de verstoorde wondgenezing verbetert door het verminderen van de ontstekingsreactie, het stimuleren van de angiogenese en het vergroten van de collageensynthese en collageenrijping. Al deze bevindingen onderstrepen het therapeutisch potentieel van de OTR4120 behandeling bij acute en vertraagd helende (chronische) wonden.

ACKNOWLEDGEMENTS

I am indebted to many people who inspired, guided, and supported me throughout the journey of completing this thesis.

First of all, I would like to deeply thank my promoter **Prof.dr. S.E.R. Hovius** for giving me the opportunity to pursue the doctoral project in his laboratory. From the moment of my PhD interview, I have always been very impressed by his incredible knowledge, sharp and quick thinking, effective decision making skills and insightful thoughts. I appreciate his support and encouragement on my research very much. It was a great honour to be his PhD student.

I would like to express my deepest gratitude to my co-promoter, **Dr. J.W. van Neck.** He has been a tremendous source of knowledge, encouragement, and inspiration. He is open-minded and has provided me not only with intellectual discussions, but also constructive advice and guidance. I appreciate the long hours and enormous efforts he spent critically reading and revising my papers. His thoughts and insights into the research were invaluable. I am also very grateful for his great personality that helped me in so many aspects of my research. Words are simply not enough to express my heartfelt gratitude for all the help and support he has given me.

My sincere gratitude also goes to the members of my doctoral committee. I appreciate **Prof.dr. E. Middelkoop, Prof.dr. P. van Zuijlen,** and **Prof.dr. J.F. Lange** for taking their time to read my thesis and provide me with very valuable feedback and advice. I thank **Prof.dr. E.P. Prens** and **Dr. M.P.M de Maat** for their participation in my doctoral committee.

I deeply thank **Prof. D. Barritault** for generously supplying me with OTR4120. I am very grateful as well for his caring about my research progress, for sharing with me his incredible knowledge, and supporting my thesis publication. I'm also inspired by his enthusiasm and unique insights in science. Furthermore, I am very grateful to **Prof.dr. D.W. van Bekkum** for providing me with the pressure ulcer model and for his hands on instruction and knowledge on the model. My deep gratitude also goes to **Dr. F. Heule** who introduced **Prof.dr. D.W. van**

Bekkum to me, and shared with me his enormous knowledge in clinical wound healing and treatment.

I am lucky to be able to work with a fine research team, whose excellent technical support and diligent work makes my thesis work possible. I am very grateful to **Bastiaan Tuk** for his commitment, contribution, and flawless work demonstrated in this thesis. His cautious and serious attitude towards experiments and excellent lab-techniques and skills in precision impressed me a lot. I also appreciate very much his kindness in helping me in so many ways during my PhD time. In addition, I thank him deeply for being my paranymph, helping and supporting me for preparing the thesis defence. Special thanks go to **Ineke Hekking,** without her, all the animal models in this thesis could not have been set up successfully. I thank her for all the excellent work, experience showed in supporting my work and the great characters in helping me all the time. I also thank **Esther Fijneman** for creating diabetes model in this thesis. It has been always a pleasure working with her who possesses positive spirit and provides valuable inputs. Many thanks also go to **Mariken Zbinden,** a former colleague who helped on setting up surgical wound and pressure ulcer models. Furthermore, I thank **Mireille Boldewijn, Mieke Pleumeekers** and **Marnix Guijt** for their contributions to the project.

My gratitude goes also to all other former and present colleagues at the Department of Plastic and Reconstructive Surgery. **Hinne Rakhorst, Sanne Moolenburgh, Dirk-Jan van der Avoort, Tim Nijhuis, Liron Duraku, Hester Kan, Antoinette van Driel, Caroline Driessen, Marjolein de kraker, Tim Damen, Dennis de Putter, Ties Molenaar, Ernst Smiths, Soledad Perez, Sandra Posthumus van Sluijs, Raul Plomp, Eveline Bijlard, Shoista Kambiz, Behnam Mohseny, Robert Dijkman, Caroline Hundepool, Jacqueline Goos,** and all other colleagues whom I have not mentioned here. I thank them for always being so kind and friendly. In particular I would like to thank the colleagues with whom I worked together. **Urville Djasim,** I'm so lucky to share the office with him. I appreciate his tremendous help in many ways, and his kindness and understanding. I thank **Wendy Tra** for being such a friendly and cooperative lab-mate. I thank **Femke Verseijden** for always being supportive and kind. I thank **Mischa Zuijdendorp** for introducing me to the breaking strength measurements. Special thanks also go to **Linda Spiegelberg** for always being helpful and being my paranymph.

I would like to thank **Carin Oostdjik** for making all the appointments to Prof. Hovius and providing the administrative support for my thesis defense.

My thesis work has involved many people from other Departments and University who provided support and help that I am deeply grateful.

Many thanks go to all the people in the laboratories of Paediatrics and Orthopaedics, with special thanks to **Theo Hoogenboezem, Ingrid Luijendijk, Rolien Raatgeep, Wilcon Smits, Janneke Bouma, Ad de Bruijn, Wendy Koevoet** and **Nicole Kops.** I thank them for always being so kind and helpful, and for sharing their experiences, instruments, and materials with me. In addition, I thank **Ram Sitaram** for providing me with MQ-water for all these years.

Sincere thanks go to **Dick Dekkers** in the laboratory of Cell Biology for his creative help and informative suggestions on Western blot analysis. I also thank **Adone Mohd-Sarip** in the laboratory of Biochemistry for her kindness and help when I used the instruments in her laboratory. I would also like to extend my thanks to **Marcel Vermeij** in the Department of Pathology who introduced me to the rat wound histology. I am very grateful for his technical assistance in histology and immunohistology and for his constructive suggestions and constant encouragement.

Many thanks go to the people in the Research laboratory of Cell Growth of Université Paris-Est in Paris, with special thanks to **Dulce Papy-Garcia, Christophe Morin, Véronique Barbier-Chassefiere, Fernando Siñeriz,** and **Patrick Kern.** I would like to thank them for their collaboration and support with the collagen biosynthesis measurements in their laboratory. In addition, my special thanks go to **Dulce Papy-Garcia**, for hosting me in her beautiful house in the first week of my work in her laboratory and for sharing her academic opinions.

Special thanks go to my Chinese colleagues at Erasmus MC. I thank **Peng Shang** for conducting PCR for me and doing it with care and an incredible efficiency. I thank **Jun Hou, Tianna Zhao, Peng Lu, Kayi Chan,** and **Elim Cheung** for their kind help and all the enjoyable chats.

I am deeply grateful for all the friendships that have blossomed here, in Holland, my second home, alongside the picturesque tulips and under the serene backdrop of the far-stretching grassland. No matter where life takes me next, there will always be a unique place in my heart for all the cherished memories I have made in this beautiful country.

I am also deeply grateful for Nanjing Medical College and Shanghai Second Medical University where I received sound education as the foundation to my current research. I am grateful as well for Nanjing University Medical School, where I gained my first work experience, many life-long friendships and joyful memories.

At last, I would like to express my deepest gratitude, in Chinese, to my family and friends in China.

爸爸妈妈, 公公婆婆, 姑父姑姑, 堂表兄弟姐妹, 以及关心我的朋友们:

谢谢你们的无限亲情和友谊。亲如水，情无价！

“昔我往矣，杨柳依依；今我来思，雨雪霏霏。”想念你们！

树范:

真爱无痕，尽在无言中。

曼曼安安:

你俩是妈妈的一壶绿茶。“碧泓如泉清甘冽，润思润神润心脾。”能喝上这样的好茶是妈妈的一种福气。妈妈会用心去品味。谢谢你们姐弟俩！

“糊涂虫”安安:

这本小书就是妈妈的“家庭作业”。这个每当你要和妈妈玩, 妈妈就会想起来的, 嚷着要去做的“家庭作业”。今天, 你的爱拖拉的妈妈 终于做完了。以后妈妈可以天天和你玩了！真的, 拉钩算数! 在这里, 妈妈想郑重地谢谢 “糊涂虫”安安对 “爱拖拉” 妈妈的忍耐和宽容。其实, 在妈妈心中, 你才是这些年妈妈收获的最大最甜的 “果子”!

童苗 鹿特丹 2012

Miao Tong, Rotterdam, 2012

CURRICULUM VITAE

Miao Tong was born on the 17th May 1966 in Nanjing, China. She studied medicine at the Nanjing Medical College between 1985 and 1990, and obtained her Bachelor degree in Medicine. She continued her Master's study in the Department of Pathophysiology at Shanghai Second Medical University, Shanghai, China, majoring in Thrombosis and Hemostasis. After obtaining her Master degree in Medicine in 1993, she had worked at Nanjing University Medical School, Nanjing, China, for 7 years as an assistant lecturer for 2 years and a lecturer for 5 years, lecturing Pathophysiology to medical undergraduate students. Meanwhile, she was a researcher investigating the effect of low level laser irradiation on immunity, a project funded by China National Natural Science Foundation. In 2000, she studied Health Care Management in the School of Management Studies for the Service Sector at the University of Surrey, Guildford, UK. In 2001, she obtained her Master degree of Science. Afterwards, she had worked as a senior production technician responsible for the custom synthesis and labelling of recombinant major histocompatibility complex peptide at ProImmune Ltd., Oxford, UK. In 2002 she moved to the Netherlands and became a research analyst on a two-year European project at the Department of Molecular Cell Biology of Leiden University Medical Centre, Leiden, the Netherlands. The subject of the project was to develop an antigen micro-array format for a serological tuberculosis assay. From May 2005 to August 2011, she had worked on the doctoral project at the Department of Plastic and Reconstructive Surgery of Erasmus MC, University Medical Center Rotterdam, under the supervision of Prof.dr. S.E.R. Hovius and Dr. J.W. van Neck. The subject of her research was wound repair and regeneration, as described in this thesis.

LIST OF PUBLICATIONS

Miao Tong, Bastiaan Tuk, Ineke M. Hekking, Esther M.G. Fijneman, Marnix Guijt, Steven E.R. Hovius, Johan W. van Neck. Diabetes-impaired wound healing is improved by matrix therapy with heparan sulfate glycosaminoglycan mimetic OTR4120 in rats. **Diabetes** (2012) 61:2633–2641.

Miao Tong; Bastiaan Tuk; Ineke M. Hekking; Mieke M. Pleumeekers; Mireille B. Boldewijn; Steven E.R. Hovius; Johan W. van Neck. Heparan sulfate glycosaminoglycan mimetic improves pressure ulcer healing in a rat model of cutaneous ischemia-reperfusion injury. **Wound Rep Reg** (2011) 19: 505–514.

Miao Tong, Bastiaan Tuk, Ineke M. Hekking, Marcel Vermeij, Denis Barritault, Johan W. van Neck. Stimulated neovascularization, inflammation resolution and collagen maturation in healing rat cutaneous wounds by a heparan sulfate glycosaminoglycan mimetic, OTR4120. **Wound Rep Reg** (2009) 17: 840–852.

Miao Tong, Mariken M. Zbinden, Ineke J. M. Hekking, Marcel Vermeij, Denis Barritault, Johan W. van Neck. RGTA OTR 4120, a heparan sulfate proteoglycan mimetic, increases wound breaking strength and vasodilatory capability in healing rat full-thickness excisional wounds. **Wound Rep Reg** (2008) 16: 294–299.

Johan van Neck, Bastiaan Tuk, Denis Barritault, **Miao Tong.** Heparan sulfate proteoglycan mimetics thrive tissue regeneration: an overview. **Tissue Regeneration** (2011) ISBN 978-953-307-876-2, Published by INTECH Open Access Publisher, http://www.intechweb.org

Cheung EY, Weijers EM, **Tong M,** Scheffer RJ, van Neck JW, Leebeek FW, Koolwijk P, de Maat MP. The effect of fibrinogen γ variants on wound healing. (Submitted)

(Major Publications before PhD Study)

Miao Tong, Catharina E Jacobi, Frans M van de Rijke, Sjoukje Kuijper, Sjaak van de Werken, Todd L Lowary, Cornelis H Hokke, Ben J Appelmelk, Nico J D Nagelkerke, Hans J Tanke, Rob P M van Gijlswijk, Jacques Veuskens, Arend H J Kolk, Anton K Raap. A multiplexed and miniaturized serological tuberculosis assay identifies antigens that discriminate maximally between TB and non-TB sera. **J Immunol Methods** (2005) 301: 1-2. 154-163.

M. Tong, Y. F. Liu, X. N. Zhao, Z. R. Hu, Z. X. Zhang. Effects of different wavelengths of low level laser irradiation on murine immunological activity and iintracellular Ca2+ in human lymphocytes and culture cortical neurogliocytes. **Lasers in Medical Science** (2000) 15(3): 201-206.

Tong, M., Zhang, Q. Significance of u-PAR, Oncoprotein C-erbB-2 and t-PA antigen expression in stomach and colon carcinoma. **Chinese Journal of Pathophysiology** (1997) 13(1): 92-95.

PHD PORTFOLIO SUMMARY

Name PhD student:	Miao Tong
Erasmus MC Department:	Plastic & Reconstructive Surgery
PhD period:	May 2005 – August 2011
Promotor:	Prof.dr. SER Hovius
Supervisor:	Dr. JW van Neck

PhD training	**Year**
In-depth courses	
• Laboratory Animal Science (University Utrecht, Utrecht, the Netherlands)	2005
• Laser Doppler Perfusion Monitoring, Laser Doppler Imager and tcpO2/pCO2 (Perimed AB, Stockholm, Sweden)	2006
• Biomedical English Writing and Communication (Erasmus MC, Rotterdam, the Netherlands)	2008
• Radiation Protection (Erasmus MC, Rotterdam, the Netherlands)	2009
(Inter) national conferences, poster presentations	
• Stimulated neovascularization and resolution of inflammation during rat cutaneous wound healing by a heparan sulfate glycosaminoglycan mimetic, OTR4120. 67th Harden - Decoding the biology of heparan sulphate proteoglycans, Robinson College, Cambridge (UK)	2009
• Diabetic ulcer healing is stimulated by synthetic Heparan sulfate mimetic, OTR4120.Tissue Engineering and Regenerative Medicine International Society (TERMIS), Granada (Spain)	2011
(Inter) national conferences, podium presentations	
• RGTA, a heparan sulfate mimetic, enhances the breaking strength during wound regeneration of rat full thickness excisional wounds. Nederlandse Vereniging voor ExperimenteleDermatologie (NVED), Lunteren (the Netherlands)	2007

- RGTA OTR4120, a heparan sulfate mimetic, improves wound regeneration of rat ischemic wounds. 12th European Conference of Scientists and Plastic Surgeons (ECSAPS) congress, Bern (Switzerland) — 2008

- OTR4120, a heparan sulfate glycosaminoglycan mimetic, improves cutaneous wound regeneration in rats. 13th European Conference of Scientists and Plastic Surgeons (ECSAPS) congress, Rotterdam (the Netherlands) — 2009

- Heparan sulfate glycosaminoglycan mimetic, OTR4120, reduces pressure ulceration and improves ulcer healing in a rat model of cutaneous ischemia-reperfusion injury. The European Tissue Repair Society (ETRS) congress, Gent (Belgium) — 2010

- Streptozotocin-induced diabetic ulcer healing is stimulated by a synthetic heparan sulfate mimetic. 14th European Conference of Scientists and Plastic Surgeons (ECSAPS) congress, Pamplona (Spain) — 2011

Teaching activities:

- Lecture wound healing,
  3rd year medical students — 2006

Award:

- One of the best oral presentations at the 14th ECSAPS congress, Pamplona (Spain) — 2011

LIST OF ABBREVIATIONS

ASC	Acid-soluble collagen
BrdU	Bromodeoxyuridine
CM	Carboxymethyl
CMB	Carboxymethyl benzylamide
CMS	Carboxymethyl sulfate
ECM	Extracellular matrix
FGFs	Fibroblast growth factors
GAGs	Glycosaminoglycans
HBGFs	Heparin-binding growth factors
HS	Heparan sulfate
HS-GAGs	Heparan sulfate glycosaminoglycans
IHC	Immunohistochemistry
IL	Interleukin
I-R	Ischemia-reperfusion
ISC	Insoluble collagen
LDF	Laser Doppler flowmetry
LDPM	Laser Doppler perfusion monitor
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
NO	Nitric oxide
NOS	Nitric oxide synthase
NPUAP	National Pressure Ulcer Advisory Panel
OD	Optical density
PDGF	Platelet-derived growth factor
PMNs	Polymorphonuclear neutrophils
PSC	Pepsin-soluble collagen
PU	Perfusion unit
RGTAs	ReGeneraTing Agents
α-SMA	Alpha-smooth muscle actin
SSC	Salt-soluble collagen

TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor α
TIMPs	Tissue inhibitors of metalloproteinases
VEGF	Vascular endothelial growth factor