Molecular and Cellular Biochemistry **116**: 197–202, 1992.

Studies on the interaction of leucocytes and the myocardial vasculature

I. Effect of hypoxia on the adherence of blood granulocytes

Anneke Pietersma, Netty de Jong, Wim Sluiter and Johan F. Koster

Department of Biochemistry I, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

Abstract

Granulytes play an important role in increasing the infarct size after ischemia and reperfusion by the release of oxygen-derived free radicals (ODFR) and lysosomal enzymes. It has been shown that the number of granulocytes adhering to the vascular endothelium increases after occlusion of the coronary artery, and that the area of myocardial damage can be reduced by preventing granulocyte adherence with monoclonal antibodies directed against adhesion receptors. The underlying mechanism of granulocyte activation under these conditions is not yet known. We have investigated whether granulocytes can be activated directly by reduced oxygen tensions. Granulocytes were suspended in a hypoxic buffer and incubated on fibronectin and gelatin coated microtitre plates at 1–3% ambient oxygen to study their ability to adhere to these matrices. The results showed that the adherence of granulocytes to fibronectin was dependent on the duration of hypoxia. After 30 min of incubation under hypoxia granulocyte adherence increased 1.3 to 1.8 fold compared to the normoxic control. The adherence to fibronectin could be inhibited partially by anti-CD18 antibody, a monoclonal antibody to the common beta chain of a class of extracellular matrix receptors. This direct activation of granulocytes due to hypoxic conditions may have implications for the interaction of these cells with the vascular endothelium *in vivo,* (Mol Cell Biochem **116:** 197–202, 1992)

Key words: granulocytes, hypoxia, adherence, CD11/CD18

Introduction

Granulocytes are important mediators of ischemia/reperfusion injury. The invasion of granulocytes into the infarcted myocard has been known for many years and was first thought of as a beneficial part of the healing process [1, 2]. Recent studies, however, demonstrate that the ultimate size of the myocardial infarct after reperfusion can substantially be reduced by depletion of blood granulocytes beforehand or by preventing the attachment of granulocytes to the target tissue with monoclonal antibodies [2].

The adherence of granulocytes to the vascular endothelium is the first step in the migration of granulocytes.

Address for offprints: A. Pietersma, Department of Biochemistry I, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

In response to chemoattractants, e.g., complement fragments and bacterial products (formylated peptides), the number of granulocyte surface receptors increases which leads to increased adherence to the endothelium and eventually diapedesis and chemotaxis [3–5]. The mechanism of granulocyte activation in ischemia/reperfusion injury is not fully elucidated but activated complement, leukotrienes, prostacyclin (PGI_2) and/or cytokines are likely to be involved [4]. Ischemic cardiac muscle releases peptides that react with the first component of complement and increased complement binding by the ischemic myocard has been shown [2]. Furthermore, leukotriene B_4 synthesized by activated granulocytes stimulates the adherence of granulocytes to the endothelium [6]. The attachment of granulocytes to the endothelium is a prerequisite for mediating vascular damage by the production of potentially histotoxic agents like proteinases and ODFR [5]. Granulocyte adherence is mediated through a class of glycoprotein receptors referred to as the CD11/CD18 complex, which are members of the integrin family of adhesion molecules [2]. The granulocyte adhesion molecules are heterodimers consisting of a common beta subunit (CD18) with different alfa subunits (CD11a, b or c) that determine the molecular specificity [2]. The ligand on the endothelial cell membrane for the CD11a/CD18 complex (lymphocyte function-associated antigen, LFA-1) is the intercellular adhesion molecule-1 (ICAM-1) that is constitutively expressed at low levels but can be markedly upregulated by the cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF) [4].

Recent investigations have shown that adherence of granulocytes to the endothelium of the ischemic myocard may be an early event in myocardial ischemia [2, 6, 7]. This led us to investigate whether hypoxia directly stimulates the adherence of blood granulocytes.

Material and methods

Experimental conditions

All incubations under hypoxic conditions were performed in a cabinet that allowed handling of cells under controlled ambient conditions. This cabinet consisted of a conducting surface coupled to a thermostatted waterbath and a hood with two holes to fit a pair of latex gloves and a small lock chamber. During incubations a continuous flow of 99.9% N_2 (N_2-gas 3.0 technical, Hoekloos, Schiedam, The Netherlands), humidified and warmed to 37° C, was maintained. The ambient pO_2 was monitored with an oxygen analyzer (type OA 250, Servomex, Zoetermeer, The Netherlands) and kept at 1–3%. Buffers were rendered hypoxic by gassing with humidified N_2 to a final pO_2 of 25 mmHg as measured by acid-base laboratory analysis (ABL3, Radiometer, Copenhagen, Denmark). Next hypoxic buffers were equilibrated to ambient conditions of 1–3% O_2.

Experimental procedures

Isolation of cells

Granulocytes were isolated pyrogen free from citrate blood obtained from healthy human donors.

Blood was diluted 1:1 with phosphate buffered saline (PBS) pH 7.4 and layered onto a Ficoll hypaque gradient (Nycomed, Haarlem, The Netherlands). After centrifugation during 15 min at 800 × g, the pellet was washed with PBS and next the erythrocytes were lysed isotonically in ammoniumchloride solution (0.155 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4). Finally, granulocytes were washed and resuspended in Krebs Ringer phosphate buffer (KRP) pH 7.4 (120 mM NaCl, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 15 mM Na_2HPO_4 and 0.1% glucose). The granulocyte fraction obtained by this procedure in general consisted for 99% of granulocytes and contained >90% viable cells as determined by trypan blue exclusion.

Incubations of granulocytes

Granulocytes were brought into the cabinet, and diluted in hypoxic buffer (1 to 5×10^5 cells/ml) and next plated onto 96-well microtitre plates (Costar, Cambridge, UK) which were coated with 5 μg fibronectin/well, isolated as previously described [8] or 5 μg gelatin/well. In a different set of experiments granulocytes (10×10^6 or 20×10^6 cells/ml) were incubated in polypropylene (PP) tubes (Greiner, Alphen a/d Rijn, The Netherlands) in KRP without calcium. After 1 h of incubation in PP tubes the number of granulocytes in suspension was counted, using a Bürkner hemocytometer, and the release of lactate dehydrogenase (LDH) from the granulocytes was measured according to the method described by Metcalf [10]. LDH release was expressed as the percentage of the total amount of LDH of a sample. All control incubations were performed under normoxic ambient conditions in a thermostatted waterbath.

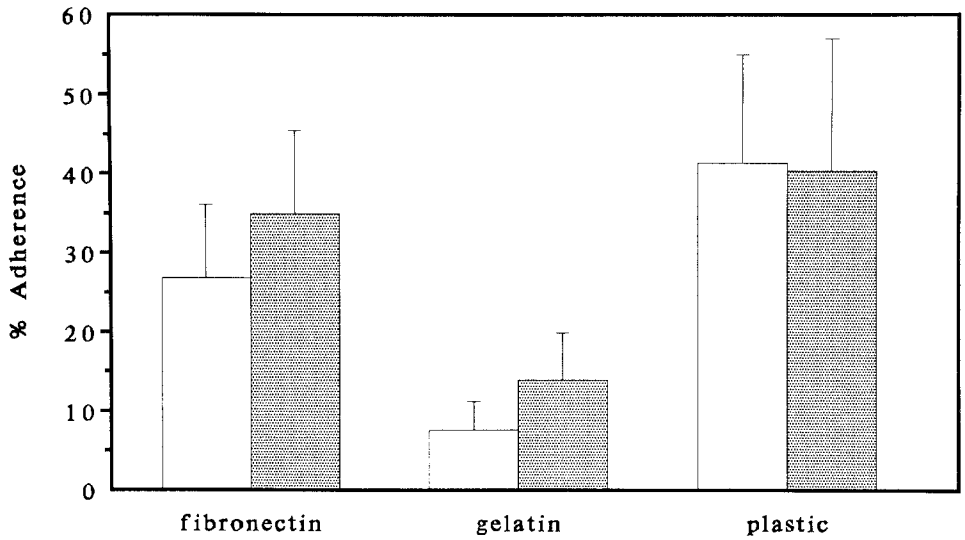

Fig. 1. Effect of hypoxia (1–3% ambient oxygen, open bars) and normoxia (21% ambient oxygen, dark bars) on the adherence of granulocytes to different substrates. Granulocytes were plated onto fibronectin and gelatin-coated or uncoated polystyrene microtitre plate wells and incubated for 30 min under hypoxic or normoxic conditions at 37° C. After extensive washing the number of adherent cells were quantitated.

Granulocyte adherence assay

After 30 min of incubation of granulocytes on fibronectin, gelatin or uncoated plastic (polystyrene) the nonadherent cells were removed by washing the microtitre plates with PBS pH 6.0. The adherent cells were quantitated according to the method described by Bath [9] using myeloperoxidase as an enzymatic marker. After lysis of the cells with 50 μl of 5% hexadecyltrimethylammoniumbromid (HTAB), 100 μl 0.2 mg/ml o-dianisidinehydrochloride), 0.4 mM hydrogen peroxide was added to each well to start the enzymatic reaction. The increase in absorption at 450 nm during 1.5 min was taken as a measure of myeloperoxidase activity. A standard curve of different concentrations of granulocytes in HTAB was used to estimate the percentage of adherent cells.

Inhibition of adhesion by monoclonal antibodies

Granulocytes were suspended at a concentration of 0.5×10^6 cells/ml in normoxic or hypoxic buffer together with various concentrations of anti-CD18 antibody (anti human LFA-1-beta1, Becton Dickinson, Etten-Leur, The Netherlands) and incubated for 30 min at 37° C in a shaking waterbath in polypropylene tubes wrapped in parafilm. Thereafter granulocytes were transferred to coated microtitre plates to study their capacity to adhere to fibronectin.

Microscopic evaluation

To allow light-microscopic examination of the adhering cells the adherence studies were repeated in fibronectin-coated (30 μg/well) 4-wells plates. After 5, 10, 20 and 30 min of incubation the wells were emptied and washed with normoxic or hypoxic KRP. Adherent cells were fixed with methanol, stained with May Grünwald-Giemsa and next counted in 5 fields of 100 mm^2 using an oculair grid.

Statistical analysis

Results are expressed as the mean and standard deviations (SD). Results from the adherence studies were analyzed with Wilcoxon's signed rank test for paired data. The results from the two other studies were analyzed using STATA (Computing Resource Centres, Los Angeles, USA) by linear regression analysis after rank transformation of the data. Results were considered statistically significant at $P < 0.05$.

Results

When granulocytes were incubated under hypoxic conditions for 1 h in PP tubes we observed a mean decrease in the number of cells of 17% (SD: 11) compared to 8% (SD: 8) for the normoxic cells. This decrease was not due to cell damage for reasons that the LDH release from cells incubated under hypoxic conditions did not differ from the normoxic control, i.e., 8.0% (SD: 3.5) and 8.5% (SD: 3.6) of total LDH, respectively. Therefore we decided to investigate whether the observed

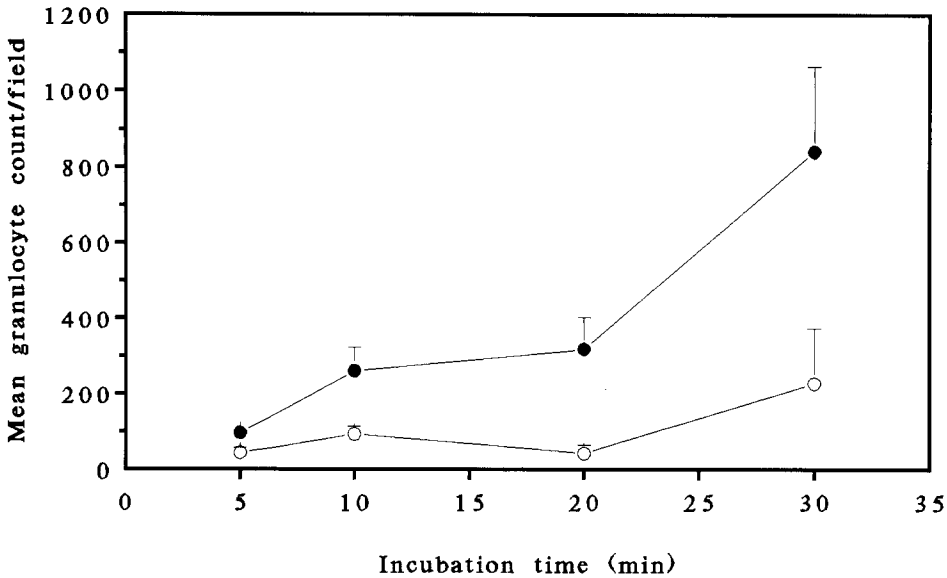

Fig. 2. The adherence of granulocytes in course of time under hypoxic (closed circles) and normoxic (open circles) conditions. Granulocytes were plated onto fibronectin coated 4-wells plates, after 5, 10, 20 and 30 min of incubation the wells were washed and adherent cells were fixed with methanol and microscopically quantitated.

decrease in the number of granulocytes after 1 h of incubation under hypoxic conditions, could be explained by an increased adherence.

Effect of hypoxia on the adherence of granulocytes to different matrices

After an incubation period of 30 min under hypoxia, granulocytes showed an increased adherence to the fibronectin matrix. The adherence of granulocytes significantly increased to 130% of their normoxic controls ($P < 0.05$; Fig. 1). Adherence of granulocytes to gelatin also significantly increased to 180% of the normoxic control ($P < 0.02$; Fig. 1). Fibronectin was found to be a better matrix for granulocytes, since adherence of granulocytes to this matrix was twice as high as to gelatin both under normoxic and hypoxic conditions. The adherence to polystyrene (uncoated microtitre plates) in normoxic and hypoxic incubations was 41.3% (SD: 13.7) and 40.3% (SD: 16.7), respectively, and exceeded binding to fibronectin, i.e., normoxia: 26.8% (SD: 9.3) and hypoxia: 34.9% (SD: 10.5), respectively. No difference due to hypoxia was observed in adherence of granulocytes to polystyrene.

To find out whether the increased adherence under hypoxic conditions is mediated through a rise in the intracellular calcium concentration, the granulocytes were incubated in calcium-free KRP with 1.5 mM ethylene glycol-bis-beta-amino ethyl ether (EGTA). The results showed that hypoxia stimulated the adherence of granulocytes to fibronectin to a similar extent in the presence or absence of calcium (130% and 150% of the corresponding normoxic control, resp.).

Effect of the duration of hypoxia on granulocyte adherence

To study granulocyte adherence in course of time, granulocytes were incubated for various time periods in 4-wells plates under normoxic and hypoxic conditions. The results presented in Fig. 2 show that the adherence of granulocytes to fibronectin under hypoxic conditions significantly increased with time ($P = 0.026$). After 30 min of hypoxic incubation the average number of granulocytes per field increased to 260% of the control value (range 160–370%). Granulocytes incubated under normoxic conditions did not exhibit a significant time-dependent increase in adherence ($P = 0.14$). In general, adherence of granulocytes was accompanied with changes in cell shape and formation of pseudopods (not shown).

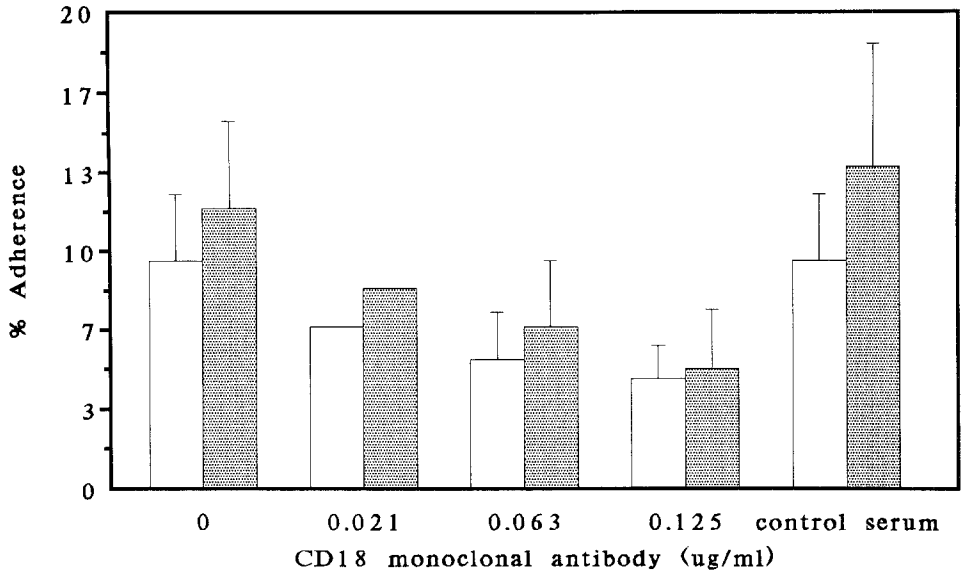

Fig. 3. Effect of blocking CD18 with anti CD18 monoclonal antibody (anti human LFA-1beta) on the adherence of granulocytes to fibronectin under normoxic (open bars) and hypoxic (closed bars) conditions. Granulocytes were preincubated with increasing concentrations of mAb for 30 min at 37° C and then plated onto fibronectin coated microtitre plate wells.

Effect of anti-CD18 antibody on granulocyte adherence to fibronectin

A significant decrease in adherence of granulocytes to fibronectin was found with increasing concentrations of anti-CD18 antibody ($P < 0.001$; Fig. 3). However, the efficacy of anti-CD18 antibody to inhibit the adherence of hypoxic granulocytes was less than that observed for normoxic granulocytes ($P < 0.01$). This indicates that another receptor that does not contain CD18 may be involved in the increased adherence of granulocytes to fibronectin under hypoxic conditions.

Discussion

The major finding of the present study was that, in comparison with normoxic granulocytes, granulocytes incubated under hypoxic conditions showed a 1.3 to 1.8 fold increase in the adherence to fibronectin and gelatin, respectively. Furthermore, the extent of adherence was dependent on the duration of the hypoxic incubation. We found that hypoxia probably directly affects granulocyte adherence.

Our results indicate that adherence in general is mediated through the CD11/CD18 class of cell surface receptors. Blocking the common beta chain of these integrin molecules with anti-CD18 antibody resulted in a significant decrease in granulocyte adherence to fibronectin for both control and hypoxic incubations. However, analysis of the decrease in granulocyte adherence by increasing amounts of anti-CD18 antibody showed that the difference between hypoxic and normoxic incubated cells remained. Conceivably the increased adherence due to hypoxia is independent of the CD18-containing class of adhesion receptors. Adherence of granulocytes to fibronectin can also be mediated by the fibronectin receptor [11]. In fact the fibronectin receptor and the CD11/CD18 complex may cooperate in the granulocyte adherence to this matrix. However, the fibronectin receptor can not be blocked by mAb to CD18 [11]. Therefore it is possible that the fibronectin receptor is responsible for the increased adherence of granulocytes due to hypoxia. Preliminary experiments indicated that depletion of intracellular calcium does not prevent the increase in adherence under hypoxic conditions. If granulocytes were depleted from intracellular calcium the difference between the adherence under hypoxic and normoxic conditions did not disappear. This indicates that the factor which causes the extra CD18-independent adherence is not mediated by the level of cytosolic calcium.

The present results are especially important in case the vessel wall is damaged during a period of ischemia/hypoxia. It is known that the subendothelial matrix contains fibronectin and collagen which is partly homologous to fibronectin [12, 13]. If during hypoxia the endothelium of the vessel wall is damaged in such a way

that the subendothelial matrix is exposed to the circulation, this could implicate increased adherence and facilitated infiltration of granulocytes into the lesion.

Acknowledgements

We thank the Dutch Heart Foundation for financial support (grant no. 89.226).

References

1. Welbourn CRB, Goldman G, Paterson IS, Valeri CR, Shepro D, Hechtman HB: Pathophysiology of ischemia reperfusion injury: central role of the neutrophil. Br J Surg 78: 651–655, 1991
2. Werns SW, Lucchesi BR: The role of the polymorphonuclear leukocyte in mediating myocardial reperfusion injury. In: Singal PK (ed.) Oxygen radicals in pathophysiology of heart disease. Kluwer Academic Publishers, Boston, Dordrecht, Lancaster, 1988, pp 123–144
3. Springer TA: Adhesion receptors in the immune system. Nature 346: 425–434, 1990
4. Ward PA, Marks RM: The acute inflammatory reaction. Curr Op Immunol 2: 5–9, 1989
5. Haslett C, Savill JS, Meagher L: The neutrophil. Curr Op Immunol 2: 10–18, 1989
6. Mullane KM, Salmon JA, Kraemer R: Leukocyte-derived metabolites of arachidonic acid in ischemia-induced myocardial injury. Fed Proc 46: 2422–2433, 1987
7. Engler R: Consequences of activation and adenosine-mediated inhibition of granulocytes during myocardial ischemia. Fed Proc 46: 2407–2412, 1987
8. Engvall E, Ruoslahti E: Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int J Cancer 20: 1–5, 1977
9. Bath PMW, Booth RFG, Hassall DG: Monocyte-lymphocyte discrimination in a new microtitre-based adhesion assay. J Immun Meth 118: 59–65, 1989
10. Metcalf JA, Gallin JI, Nauseef WM, Root RK: Laboratory manual of neutrophil function. Ravenpress, New York, 1986, pp 171–172
11. Nathan C, Srimal S, Farber C, Sanchez E, Asch A, Gailit J, Wright SD: Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. J Cell Biol 109: 1341–1349, 1989
12. Hynes RO: Integrins: A family of cell surface receptors. Cell 48: 549–554, 1987
13. Yamada KM: Adhesive recognition sequences. J Biol Chem 266: 12809–12812, 1991