Hydrocortisone-Induced Increase of PDGF β-Receptor Expression in a Human Malignant Mesothelioma Cell Line

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The effect of hydrocortisone (HC) on PDGF β-receptor expression was studied in the human malignant mesothelioma cell line Mero-14. HC was found to induce a time- and dose-dependent increase of PDGF β-receptor mRNA. Nuclear run off analysis revealed that HC induced increased transcription of the PDGF β-receptor gene. The expression of PDGF β-receptor protein was also elevated by HC as demonstrated with an immunoblotting assay. However, the number of PDGF–BB binding sites on the cell surface of Mero-14 remained unchanged upon HC treatment. These results suggest that steroid hormones can regulate PDGF receptor expression *in vivo.* © 1992 Academic Press, Inc.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a mitogen for fibroblasts, smooth muscle cells, and glial cells and is composed of two disulfide-bonded polypeptides denoted PDGF A-chain and PDGF B-chain [1, 2]. Three possible isoforms of PDGF (AA, AB, and BB) have been identified [3–5].

Two distinct PDGF receptors, designated PDGF α- and PDGF β-receptor, were identified [6, 7] and found to dimerize upon binding of PDGF [8–10]. This can result in three distinct PDGF receptor dimers (αα, αβ, and ββ) with different specificities for PDGF: αα-receptors can be formed by all isoforms, αβ-receptors by PDGF-AB and PDGF-BB, and ββ-receptors by PDGF-BB [10, 11]. The PDGF α- and β-receptors have been cloned and found to be encoded by two different genes mapped to human chromosomes 4 and 5, respectively [12–17].

The type of PDGF receptor that is expressed and the level of this expression could be the decisive factor in PDGF responsiveness. In several chronic inflammatory conditions like psoriasis, atherosclerosis, and rheuma-

toid arthritis induction of PDGF β-receptor expression was found on vascular and connective tissue cells [18–21]. The mechanism of regulation of PDGF receptor expression is still unknown. So far, few data are available on the regulation of PDGF receptor expression. Gronwald et al. [22] found that TGF-β decreased the number of PDGF α-receptors and increased the number of PDGF β-receptors in 3T3 cells. A TGF-β-induced decrease in PDGF α-receptor expression was also reported for human fibroblasts and smooth muscle cells [23]. In addition, PDGF itself induces the synthesis of the PDGF β-receptor [24]. Embryonal carcinoma cells were found to increase the transcription of the PDGF α-receptor gene upon exposure to retinoic acid with or without dibutyryl cyclic AMP [25]. Furthermore, it was recently suggested that the synergistic effect from combinations of cyclic AMP and growth factors on Schwann cells may be due to cyclic AMP-mediated induction of growth factor receptors [26].

Human malignant mesothelioma is a mesodermally derived tumor most frequently found in the pleura. Human malignant mesothelioma cell lines were found to express PDGF β-receptors, whereas cultured normal mesothelial cells express predominantly PDGF α-receptors [27]. Furthermore, expression of PDGF A- and B-chain mRNA was found in malignant mesothelioma cell lines, while normal mesothelial cells expressed PDGF A-chains and had a barely detectable level of PDGF B-chain mRNA [28, 29].

In this paper we describe a time- and dose-dependent increase of PDGF β-receptor mRNA expression upon addition of hydrocortisone (HC) to the culture medium of the malignant mesothelioma cell line Mero-14. In addition, we studied whether the PDGF β-receptor mRNA induction was due to increased transcription or differences in RNA stability. Immunoblotting and binding experiments with 125I-PDGF were performed to investigate the effect of HC on the protein level.

MATERIALS AND METHODS

Cell lines and growth conditions. The isolation of normal and malignant mesothelial cell lines and the characteristics of Mero-14 were described earlier [30, 31].

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Cells were cultured in F10 medium with 15% fetal calf serum (FCS), 0.1 mg/ml streptomycin sulfate (Biochrom KG, Berlin, Germany), and 100 IU/ml penicillin G sodium (Gist-Brocades, Delft, The Netherlands). The medium was supplemented with 10 ng/ml epidermal growth factor (EGF; Collaborative Research, Lexington, MA) and/or 0.1, 0.2, 0.4, 0.8, or 1.6 μg/ml hydrocortisone (Pharma Chemie, Haarlem, The Netherlands) when indicated.

RNA isolation and Northern blot analysis. RNA isolation and Northern blot analysis were performed as described [29]. The filters were washed at 42°C to 0.3× SSC and exposed to a Fuji-RX film.

Probes. The PDGF α-receptor probe was a 1.5-kb *Eco*R1 fragment [13] and the PDGF β-receptor probe was a 1-kb *Pst*1 fragment [12]. These fragments correspond to the extracellular domain of the PDGF α- and PDGF β-receptors, respectively. The GAPDH probe was a 0.7-kb *Eco*R1–*Pst*1 fragment [32].

PDGF, radiolabeled ligands, and radioreceptor assay. PDGF-AA and PDGF-BB were recombinant material expressed in yeast and were ^{125}I-labeled by the chloramine T and Bolton and Hunter methods, respectively, to specific activities of 80,000 (PDGF-AA) and 50,000 (PDGF-BB) cpm/ng [33].

For the radioreceptor assay, cells were plated in 12-well plates (Costar, Cambridge, MA). When confluence was reached, the cells were washed with phosphate buffered saline (PBS) and incubated for 24 h in serum-free F10 medium at 37°C. Prior to binding, the cells were washed with 1 ml PBS supplemented with bovine serum albumin (BSA; Boehringer, Mannheim, Germany), 1 mg/ml. Binding was performed for 2 h at 0°C in a volume of 350 μl PBS–BSA containing 50,000 cpm radiolabeled ligands with or without unlabeled PDGF (250 ng/ml). After three washes with ice-cold PBS–BSA the cells were lysed in 2% Triton X-100 in H$_2$O. ^{125}I-Radioactivity was counted in a gamma counter.

Nuclear run off assay. Nuclei were isolated from 10^8 cells essentially according to Zenke *et al.* [34] with the presence of 0.5% NP-40 in the lysis buffer as the only modification. From each cell line 1–2×10^7 nuclei were used for a nuclear run off analysis, as adapted from Linial *et al.* [35]. The ultimate concentration of Tris–HCl, pH 8.0, in the run off buffer was 6 mM, while 140 μCi [α-^{32}P]UTP was added. Synthesis of transcripts took place at 30°C for 20 min followed by treatment with 10 μg DNase I for 5 min at 30°C. After centrifugation, the nuclear RNA pellet was resuspended in a buffer containing 45% (v/v) formamide, 0.2 M NaP$_i$ (pH 7.2), 1 mM EDTA, 7% SDS, and 250 μg/ml yeast RNA.

Plasmid DNA was spotted on nitrocellulose filters with the Schleicher and Schuell slot blot apparatus (S & S, Dassel, Germany) and immobilized for 2 h at 80°C. Subsequently, the filters were hybridized with the labeled RNA for 2 days at 45°C. After hybridization, filters were washed for 8 h at 65°C in 40 mM NaP$_i$ (pH 7.2), 1% SDS interrupted by washing in 2× SSC containing RNaseA (5 μg/ml) for 30 min at 37°C. Autoradiography was performed at −80°C with Kodak XAR films.

Immunoblotting. Mero-14 cells were cultured until confluence was nearly reached either without HC or with the addition of 0.4 or 0.8 μg/ml HC. Cells were washed with PBS and harvested in PBS with 0.02% NaEDTA. For each lane 2 × 10^6 cells were lysed in a buffer containing 0.5% Triton X-100, 0.5% deoxycholate, 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, and protease inhibitors. After centrifugation, the lysate was incubated with wheat germ lectin Sepharose 6 MB (WGA; Pharmacia, Uppsala, Sweden) on an end-over-end rotator overnight at 4°C. After washing with lysis buffer the beads were boiled for 5 min in sample buffer containing 2.5% SDS. The proteins were separated on a 8% SDS–polyacrylamide gel and blotted to nitrocellulose. Subsequently, the filter was washed twice in PBS blocked in skim milk and washed three times in PBS, containing 0.2% Tween. The filter was incubated 2 h at room temperature in 1:100 diluted PDGFR-3, which recognizes PDGF β-receptors [36].
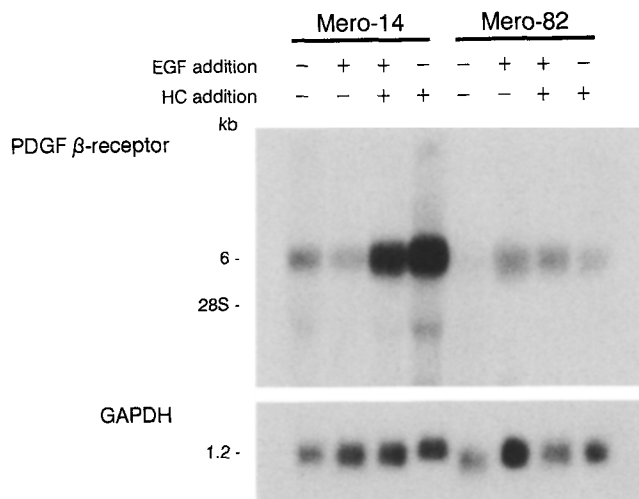

FIG. 1. Northern blot analysis of 25 μg total RNA from the malignant mesothelioma cell lines Mero-14 and Mero-82. These cell lines were cultured for 24 h in medium with 15% FCS and supplemented with EGF (10 ng/ml), EGF (10 ng/ml) plus HC (0.4 μg/ml), or HC (0.4 μg/ml) only. The filter was hybridized to a ^{32}P-labeled PDGF β-receptor probe and a GAPDH probe.

After washing, goat anti-rabbit-IgG-alkaline phosphatase (Tago, Burlingame, CA) was used as a second step.

Growth kinetics. Triplicate cultures were started in 12-well plates (25 × 10^3 cells/culture). After 24 h the medium was changed to F10 medium with 15% FCS and 0.4 μg/ml HC or F10 medium with FCS only. Two days later, the cells were harvested by trypsinization and the number of cells was counted.

RESULTS

Effect of Hydrocortisone on PDGF β-Receptor mRNA

According to Connell and Rheinwald [37] normal mesothelial cells have an optimal growth in medium supplemented with EGF and HC. For our experiments concerning PDGF receptor expression, normal mesothelial cells were cultured in medium supplemented with EGF and HC, whereas the malignant mesothelioma cell lines did not require the addition of EGF and HC. In order to investigate whether addition of these supplements affected the PDGF receptor expression in normal and malignant mesothelial cells, seven malignant mesothelioma cell lines were cultured with EGF and HC and normal mesothelial cells without. No difference in the pattern of PDGF receptor expression of normal and malignant cells was observed, except for Mero-14. This malignant mesothelioma cell line was found to increase the level of PDGF β-receptor mRNA expression upon addition of EGF and HC to the culture medium (Fig. 1). The effect of EGF only and HC only on PDGF β-receptor expression in Mero-14 was studied and compared with the malignant mesothelioma cell line Mero-82. These cell lines were cultured for 24 h in F10 medium with 15%
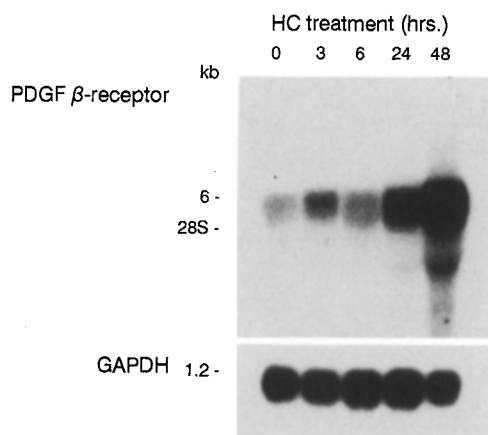
FIG. 2. Northern blot analysis of 25 μg total RNA of Mero-14 cells incubated with HC (0.4 μg/ml) for 3, 6, 24, or 48 h. The filter was hybridized to a ^{32}P-labeled PDGF β-receptor probe and a GAPDH probe.
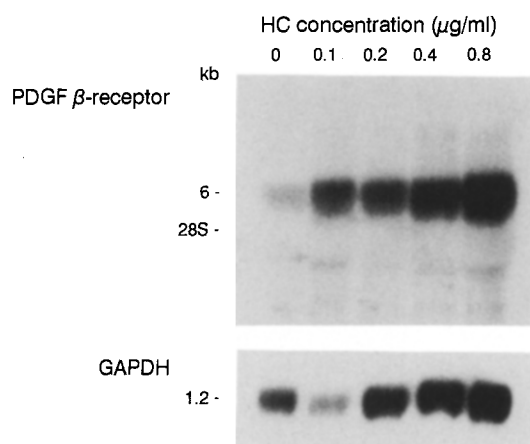

FIG. 3. Northern blot analysis of 25 μg total RNA of Mero-14 cells incubated for 24 h with various HC concentrations (0.1, 0.2, 0.4, and 0.8 μg/ml). The filter was hybridized to a ^{32}P-labeled PDGF β-receptor probe and a GAPDH probe.

FCS and supplemented with EGF, EGF plus HC, or HC only. After harvesting, RNA was isolated and analyzed for PDGF β-receptor RNA expression. Mero-14 showed an increase in PDGF β-receptor mRNA upon the addition of EGF plus HC or HC only (Fig. 1). Addition of EGF only to Mero-14 cells resulted in a similar level of PDGF β-receptor mRNA expression as F10 medium without supplementation (Fig. 1). Mero-82 was used as a control and did not exhibit a change in PDGF β-receptor RNA expression upon addition of EGF and/or HC to the culture medium (Fig. 1).

To determine the time dependence of the increase of PDGF β-receptor mRNA expression in Mero-14, mRNA was prepared from cells incubated with HC for 3, 6, 24, or 48 h. HC caused an increase of PDGF β-receptor mRNA in Mero-14, which was clearly detectable from 24 h after incubation with HC and was even more pronounced after 48 h (Fig. 2).

Northern blot analysis of mRNA from Mero-14 incubated with various concentrations of HC revealed a dose-dependent increase in PDGF β-receptor mRNA expression from 0.1 to 0.8 μg/ml HC (Fig. 3). In addition to HC, dexamethasone was found to increase the PDGF β-receptor mRNA expression in Mero-14 (data not shown). Hybridization of a filter with mRNA from Mero-14 and several other malignant mesothelioma cell lines with a probe for the glucocorticoid receptor revealed the presence of mRNA for this receptor in Mero-14 as well as in other malignant mesothelioma cell lines at approximately similar levels (data not shown).

In order to investigate whether the observed HC-induced increase of PDGF β-receptor mRNA was due to an increased rate of transcription or a difference in cytoplasmic degradation, nuclear run off analysis was performed. Nuclear RNA levels of PDGF A- and B-chain and PDGF α- and β-receptor in HC-treated Mero-14 cells were compared with those in untreated Mero-14 cells (Fig. 4). The quality and expression of the PDGF receptor RNA was related to the constitutively expressed actin gene and pUC was used to exclude the possibility that the observed signal was due to cross hybridization with vector sequences. These experiments revealed that HC-treated Mero-14 cells had an elevated level of nuclear PDGF β-receptor RNA compared to that of untreated Mero-14 cells. The PDGF α-receptor mRNA remained undetectable in HC-treated and untreated Mero-14 cells.

Effect of HC on PDGF β-Receptor Protein

To investigate whether the observed increase in PDGF β-receptor mRNA upon HC addition results in an elevation of the amount of PDGF β-receptor protein,
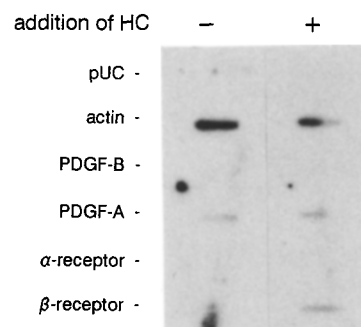

FIG. 4. Nuclear run off analysis of ^{32}P-labeled nuclear RNA of Mero-14 cells cultured with or without HC. The radiolabeled transcripts were hybridized to the plasmids pUC, pUC + actin, pUC + PDGF-B, pUC + PDGF-A, pUC + PDGF α-receptor, and pUC + PDGF β-receptor.
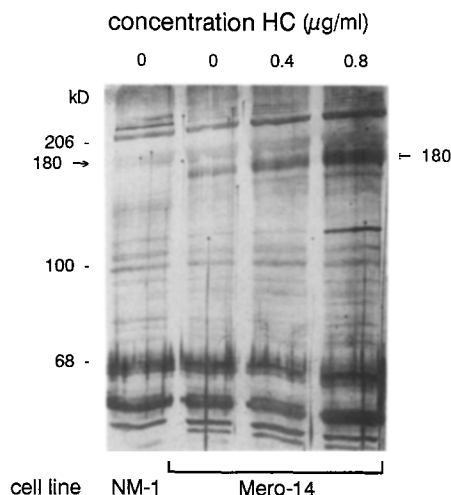
concentration HC (µg/ml)

0   0   0.4   0.8

FIG. 5. Detection of PDGF β-receptors by immunoblotting with the antibody PDGFR-3 in Mero-14 cells cultured for 24 h without HC or with 0.4 or 0.8 µg/ml HC. As a control the PDGF β-receptor negative normal mesothelial cell line NM-1 was used.

immunoblotting and radioreceptor assays were performed.

For immunoblotting, Mero-14 cells were incubated for 24 h either without HC or with 0.4 or 0.8 µg/ml HC. The normal mesothelial line NM-1 which has no detectable PDGF β-receptors was used as a control. After lysis and incubation with WGA–Sepharose, the glycoprotein-enriched lysate was separated by electrophoresis and blotted. The filter was incubated with the PDGF β-receptor specific antiserum PDGFR-3. As a control, lanes were incubated with normal rabbit serum and PBS. In all three samples of Mero-14 cells, a PDGF β-receptor specific band of 180 kDa was detected. This band increased in intensity upon the addition of increasing amounts of HC prior to lysis, showing the most pronounced band when Mero-14 cells were incubated with 0.8 µg/ml HC (Fig. 5). Control lanes did not reveal a PDGF β-receptor specific band. These data indicate that HC increases not only PDGF β-receptor mRNA but also PDGF β-receptor protein synthesis in Mero-14.

The level of membrane-associated PDGF β-receptors in Mero-14 and Mero-82 cells cultured with and without HC was compared using a radioreceptor assay with ^{125}I-labeled PDGF-AA and ^{125}I-labeled PDGF-BB. The binding of ^{125}I-labeled PDGF-AA to Mero-14 and Mero-82 cultured without HC was not significantly over the background. ^{125}I-labeled PDGF-BB was found to have a specific binding to Mero-14 and Mero-82, which was lower in the presence of unlabeled PDGF-BB (Fig. 6). These results are in agreement with earlier experiments with a panel of malignant mesothelioma cell lines [27].

Addition of 0.4 µg/ml HC to Mero-14 and Mero-82 24 h prior to binding experiments did not reveal an increase in the specific binding of either ^{125}I-labeled

PDGF-AA or ^{125}I-labeled PDGF-BB compared to cells cultured without HC. These results suggest that the increase in synthesis of PDGF β-receptor protein expression after 24 h HC treatment does not result in a net increased cell surface expression of PDGF β-receptors. An alternative possibility is that HC causes also a decrease in receptor affinity. This has not been excluded.

Proliferation of Malignant Mesothelioma Cell Lines in the Presence of HC

We investigated whether the addition of HC also affected the proliferation of malignant mesothelioma cell lines. Four malignant mesothelioma cell lines were grown in the presence of 0.4 µg/ml HC for 48 and 72 h, followed by counting of the cell numbers. The proliferation of all four investigated malignant mesothelioma cell lines (Mero-14, -41, -48b, and -82) was found to be inhibited compared to cells grown without HC. The percentage inhibition after 48 and 72 h varied between 20 and 31 (Table 1).

DISCUSSION

This paper shows that HC enhances the PDGF β-receptor mRNA and protein synthesis in the human malignant mesothelioma cell line Mero-14 in a time- and dose-dependent manner. Nuclear run off analysis revealed that the HC-induced effect on PDGF β-receptor mRNA expression was due to an increased transcription rate of the PDGF β-receptor gene. In contrast, PDGF α-receptor mRNA was undetectable with or without HC addition.

In a previous paper we described expression of the PDGF A- and B-chain genes in a panel of malignant mesothelioma cell lines including Mero-14 [32]. These cell lines were found to express PDGF β-receptors [25]. Coexpression of PDGF B-chain and PDGF β-receptors is thus common in malignant mesothelioma cell lines and suggests that autocrine growth stimulation involving PDGF may be important in the transformation process. In the case of the Mero-14 cell line, HC stimulation might thus, under certain conditions, potentiate an autocrine loop, by stimulation of PDGF β-receptor synthesis. HC treatment did not affect the PDGF B-chain mRNA expression in Mero-14 cells (data not shown).

The effect of HC on PDGF β-receptor expression in Mero-14 was detected on the mRNA and protein level, whereas no increase of membrane bound receptors was found in the receptor binding assay. It should be noted, however, that the amount of PDGF receptors at the cell surface is dependent on the balance between synthesis and degradation of receptors. In Mero-14 cells, the degradation is likely to be accelerated by the endogenous production of PDGF, which is likely to induce an increased internalization and degradation of receptors:
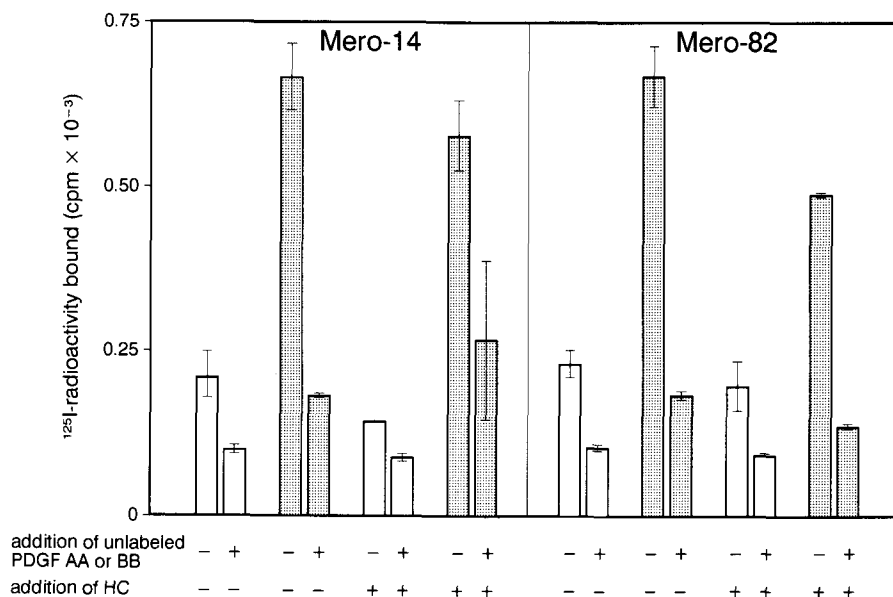
FIG. 6. Binding of ^{125}I-PDGF-AA (open bars) and ^{125}I-PDGF-BB (stippled bars) in the absence (−) or presence (+) of unlabeled ligand to Mero-14 cells cultured for 24 h without (−) or with HC (+) (0.4 μg/ml).

whether the degradation rate is affected by HC treatment remains to be determined.

Glucocorticoids have been shown to stimulate or inhibit cell proliferation in a variety of cell culture systems. We demonstrated that HC inhibited the growth of four human malignant mesothelioma cell lines including Mero-14. The mechanism for inhibition remains to be elucidated. Clearly, however, HC affects growth of Mero-14 cells in more than one way; whereas the induction of PDGF β-receptors would be expected to stimulate growth, the net effect during the *in vitro* conditions studied in this communication is growth inhibition.

HC is a glucocorticoid that, upon binding to the glucocorticoid receptor, regulates transcription of genes that have a glucocorticoid responsive element (GRE) [38]. Since the promoter region of the PDGF β-receptor gene has not yet been characterized, it is not known

TABLE 1

Inhibition of the Proliferation of Malignant Mesothelioma Cell Lines upon Addition of HC to the Culture Medium

Cell line	Culture period	
	48 h^a (%)	72 h^a (%)
Mero-14	22	25
Mero-41	25	30
Mero-48b	31	20
Mero-82	28	30

^{a} At 48 and 72 h after addition of HC the cells were harvested and counted. Data presented are the mean of two independent experiments.

whether it contains a GRE. It was suggested earlier by Terracio *et al.* [39] that the observed expression of PDGF β-receptor on cells of the endometrium may be steroid hormone dependent. Although it cannot be excluded that the effect of HC is directly on PDGF-β receptor transcription, an indirect effect seems more likely. This is supported by the observation that 24 h HC treatment is necessary for a detectable increase in PDGF β-receptor mRNA, which is rather long for a direct effect of HC on PDGF-β receptor gene expression. For the insulin receptor, for instance, it has been described that HC can induce an increase in specific receptor mRNA within 2 h [40]. Furthermore, it was recently reported by Wang *et al.* [25] that embryonal carcinoma cells after retinoic acid addition increase their PDGF α-receptor RNA expression within 4 h [25].

From seven investigated malignant mesothelioma cell lines only Mero-14 showed increased PDGF β-receptor expression upon culturing in medium supplemented with HC. It is unclear why of seven investigated malignant mesothelioma cell lines that all express PDGF β- and glucocorticoid receptors, only Mero-14 cells increased their PDGF β-receptor expression upon HC addition. This may suggest that a cofactor is involved which is activated in Mero-14 cells only. All malignant mesothelioma cell lines have abnormal karyotypes with multiple numerical and structural abnormalities [30, 31]. Comparison of the karyotype of Mero-14 with the other malignant cell lines did not reveal an aberration that could be involved in the HC-induced PDGF β-receptor expression.

In conclusion, we have demonstrated that HC induces an increase of PDGF β-receptor mRNA and protein in

the malignant mesothelioma cell line Mero-14. This increase was found to be due to an elevated transcription of the PDGF β-receptor gene. Our results suggest that regulation of PDGF receptor expression *in vivo* may be elicited by steroid hormones.

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