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Extracellular matrix proteins influence phenotype and cytokine expression in human breast cancer cell lines

Abstract

Tumor growth and invasion are not only the result of malignant transformation but are also dependent on environmental influences from surrounding stroma, extracellular matrix (ECM), local cytokines and systemic hormones. We have investigated the influence of ECM components on three human breast cancer cell lines of different malignant potential: MCF-7, T47D and MDA-MB-231 were

cultured on collagen I, collagen IV, laminin, fibronectin or poly-D-lysine, and we analyzed the proliferation rate and cytokine expression pattern. Among the three cell lines investigated we observed a distinct response to each ECM component. We hypothesize that ECM may have a significant modulatory effect on malignant behavior in vivo which might depend on individual responses and on the differentiation state of tumor cells. This study also shows that the surface on which cells are cultured greatly influences cell kinetics and the cytokine expression pattern.

Full text

INTRODUCTION

Tumor growth and invasion are not caused by malignant cells alone, they are also greatly dependent on a complex system of auto-, para- and juxtacrine interactions [1]. Systemic hormones, local growth factors and cytokine-mediated interactions between cancer cells and their surrounding stroma play an important part in maintaining tissue architecture [2]. Moreover, tumor cell growth and metastatic behavior are also influenced by various extracellular matrix (ECM) components and basement membrane proteins [3].

ECM is a key component of the cellular microenvironment and consists of several proteinous and non-proteinous components, which play a central role in determining and influencing cellular phenotype [1]. These structures provide the spatial orientation and the stability required for the organization and development of tissue-specific histology. Collagen I forms the structural backbone of the matrix and is part of the framework that holds cells and tissues together, where as fibronectin and laminin are part of the stabilizing scaffolding which links connective tissue cells to each other and connects connective tissue cells with parenchymatous cells *via* basement membranes. Collagen IV is found in basement membranes, the sheet-like matrix which underlies epithelial and endothelial cells [2].

However, ECM is not merely an inert scaffolding, it is an essential player in the regulation of cell growth and differentiation [4, 5]. Furthermore, ECM components participate actively in the basic dynamic processes such as cell attachment, spreading and migration [2].

There are indications that ECM stimulates tumor cells to secrete different kinds of growth factors, growth inhibiting factors and cytokines [3]. Although poly-D-lysine is a synthetic molecule and not part of the ECM-protein skeleton *in vivo*, it is widely used *in vitro* to enhance cell attachment to plastic or glass surfaces. These positively charged polymers are claimed (by the manufacturers) not to stimulate biological activity. Therefore, we also investigated the influence of this adhesive factor on breast cancer cells phenotype and cytokine expression pattern.

We have analyzed the cytokines bFGF, VEGF, TGF-beta1, IL-1alpha, endostatin and EGF, which are known to be expressed in breast cancer and which are influenced by ECM components [6-14]. To mimic *in vivo* cell-matrix interactions, we used a cell-culture system which attempts to simulate the ECM environment in the human breast.

Changes in cell number (increase as opposed to decrease), morphology and cytokine expression pattern were investigated in three human breast cancer cell lines, which were cultured on collagen I, collagen IV, laminin, fibronectin or poly-D-lysine under serum-free and hormone-free conditions. We used these well-defined, purified molecules, and not matrigel, to achieve reproduceable and clear-cut data. Matrigel, for example, is a solubilized basement membrane matrix extracted from the EHS (Engelbreth-Holm-Swarm) mouse tumor. It also contains growth factors, matrix metalloproteinases, and other proteinases, as well as several undefined compounds of non-human origin, which might interfere with our ELISA or lead to false positive or negative results.

In order to determine whether a differential response was induced in cells of varying malignant potential, we chose three human breast cancer cell lines which differ from each other in their estrogen and progesterone receptor (ER and PR) status and in invasive behavior: MCF-7, a sex-steroid receptor-positive cell line which has retained several characteristics of differentiated mammary epithelium, including ability to process estrogen signaling and the capability of forming domes, MDA-MB -231, a highly anaplastic cell line without ER and PR, and T47D, a cell line with intermediate malignant behavior, containing cytoplasmatic junctions and receptors to 17 beta-estradiol and progesterone [15-18].

MATERIALS AND METHODS

Cell lines and culture conditions

All cell lines were supplied by the ATCC (Rockville, MD, USA). The human breast cancer cell lines MCF-7 and MDA-MB-231 were grown in Dulbecco's minimum essential medium (DMEM) high glucose (4.5 g/l) with phenol red. T47D was maintained in RPMI 1640 medium with 2 mM glutamine. All media were supplemented with penicillin (50 units/ml), streptomycin (50 mug/ml), bovine insulin (10 mug/ml for MCF-7 and T47D, Gibco, USA), and 10 % fetal calf serum (FCS). Cell lines were cultivated in 75 cm^2 flasks at 37° C in a humidified atmosphere of 5% CO_2, and subcultured weekly after detachment with 0.05% trypsin until experiments were performed.

ECM assays

Cells were plated in 6-well culture dishes, which were either untreated (plastic, Costar, USA) or coated with collagen IV, laminin, h-fibronectin, collagen I or poly-D-lysine, with serum-containing medium overnight. Coated culture dishes were purchased from Becton Dickinson, USA (BioCoat^{TM} 6-well Variety Cellware Packs). Cells were cultured in FCS-containing medium until 70%-80% confluence, which was usually within 12-16 hours. Cells were then washed with phosphate-buffered saline (PBS) twice, and ITS-enriched (5 mug insulin/5 mug transferrrin/5 mug sodium selenite/ml medium; Sigma, USA) serum-free medium without phenol red was added (= day 0). ITS offers the opportunity to work in a serum-free culture environment to avoid the variability and contaminants that can be introduced by serum. Bovine insulin, human transferrin and sodium selenite are used by most mammalian cells and help the cells to survive during the 48 hour-assays in the absence of FCS and hormones [19, 20]. After 48 hours (= day 2), supernatants were collected and immediately frozen - 20° C. The attached cells were trypsinized and counted on day 2 on a Bürker Türk`s counter chamber.

Cell morphology was documented by photography at day 2 with x 40 magnification. (Camera Olympus inverted research microscope model IMT-2 with 100 W Halogen illuminator).

Cytokine detection

Cytokine and growth factor release into cell culture supernatants was measured by ELISA kits: h-IL-1alpha, h-bFGF and h-endostatin Accucyte kits were purchased from Cytimmune Sciences inc., College Park, Maryland, USA. H-VEGF and h-EGF Quantakine ELISA were bought from R & D Systems, Minneapolis, USA. The TGF-beta1 kit was purchased from DRG Instruments GmbH, Germany. All assays were performed according to the manufacturer's instructions.

RESULTS

Tumor cell number is influenced by ECM proteins

In comparison to the uncoated plates, MCF-7 plated on collagen IV and I showed a 2.5 fold and a 1.5 fold increase in cell number on h-fibronectin and poly-D-lysine, respectively. Conversely, on laminin, cells were reduced to 5% of the original cell count (Figure 1, panel A). T47D displayed a different growth pattern on h-fibronectin and poly-D-lysine than MCF-7, since after 48 hours only 70% of the originally plated cells were counted. Growth on collagen IV was comparable to the uncoated, whereas collagen I increased tumor cell number 3.2 fold. Adhesion behavior on laminin was comparable to MCF-7 (Figure 1, panel B). MDA-MB-321 displayed a rather moderate proliferation on collagen IV and a 1.2 fold cell propagation on h-fibronectin and collagen I. Within 48 hours cells were reduced to 24% and 5.7% on poly-D-lysine and laminin, respectively, whereas on the uncoated plates 76% of the original cell number was counted (Figure 1, panel C).

Cell morphology is dependent on cell-matrix interactions

Phenotypical behavior of MCF-7 was documented by photography at the end of the 48 hours assay. Since the "overall pattern" of cellular morphology looked similar in all three cell lines, results are shown for MCF-7 only (Figure 2, A-F). Whereas all three cell lines grew to confluence on h-fibronectin (D), collagen I (B) and IV (F) in a dense monolayer lawn, the most striking differences in morphology were observed when cells were plated on laminin (E) and poly-D-lysine (C): these two surfaces did not allow the cells to grow in monolayers. Cultured on laminin, cells formed clusters and detached from the bottom of the culture dishes. Spheroids began to float in the supernatant within the 48 hours of the assay. When plated on poly-D-lysine, cells formed three-dimensional reticular structures. The tendency to detach from the surface was not observed to the same extent (data not shown).

Cells growing on uncoated plates (A) formed monolayers, but developed vacuoles indicating a lack of growth.

Cytokine expression pattern is influenced by ECM

The expression pattern of TGF-beta1 in MCF-7, T47D and MDA-MB-231 in result to different ECM components is shown in Figure 3A: significant levels of TGF-beta1 were produced by MDA-MB-231 when plated on poly-D-lysine and laminin (10.7- and 5-fold in comparison to the uncoated, respectively). No significant differences were observed between collagen I, collagen IV and h-fibronectin. Interestingly, the two poorly invasive cell lines, MCF-7 and T47D, did not express significant levels of TGF-beta1 on any of the ECM-coated plates. The expression pattern of IL-1alpha is shown in Figure 3B: laminin was the strongest stimulator for IL-1alpha release in all three cell lines. Laminin induced a 7.2-fold, 140-fold and a 50-fold release of this cytokine in MDA-MB-231, T47D and MCF-7, respectively. Il-1alpha release was 22 fold (550 pg/ml/10^5 cells) higher in MDA-MB-231 plated on poly-D-lysine. Il-1alpha release from all three cell lines cultured on plastic, collagen I, collagen IV and fibronectin was low, at between 5 and 30 pg/ml/10^5 cells. The release of h-VEGF is described in Figure 3C: MDA-MB-231 grown on plastic expressed a steady state, with 170 pg/ml/10^5 cells. No significant change in VEGF-release could be observed on collagen I, collagen IV and fibronectin, whereas a 3-fold induction was detected on poly-D-lysine. No alterations were observed in MCF-7 and T47D grown on any of the ECM-coated plates. The expression pattern of bFGF is shown in Figure 3D: no bFGF was detected in any of the three cell lines plated on uncoated dishes. T47D was the only cell line in which bFGF secretion was consistently stimulated upon growth on h-fibronectin and laminin, with 600 and 1,600 pg/ml/10^5, respectively. Collagen IV was the only ECM component which stimulated MDA-MB-231 to release bFGF (220 pg/ml/10^5 cells). MCF-7 plated on collagen I displayed a weaker response with 60 pg/ml/10^5 cells. We also investigated the release of h-endostatin and h-EGF from the three cell lines cultured on the biocoats mentioned, but no measurable amounts of proteins were detected by ELISA (data not shown).

DISCUSSION

Tumor invasion is highly dependent on and influenced by its microenvironment, including stroma, ECM and local cytokines [21]. In carcinomas, the cellular organization is dramatically changed and tumor stroma is extensively modified.

The basement membrane is penetrated in a process of degradation and direct contact between tumor cells and surrounding stroma, which coincides with neovascularization and remodeling of the extracellular matrix. Stroma-derived growth factors, such as insulin-like growth factor I and II (IGF-I and II), are strong mitogens for breast cancer epithelium [22, 23]. These data reflect the importance of paracrine influences mediated by soluble *in vivo* factors.

The role of ECM- and breast cancer cell-derived cytokines, however, is still largely unknown. We have therefore tried to elucidate aspects of these complex interactions between tumor cells and their matrix. Our results show that tumor cell lines of different malignant potential display a heterogeneous response to ECM components as measured by the expression pattern of several cytokines.

It is widely reported that a breast carcinoma *in vivo* shows progression from a hormone-dependent growth to a hormone-independent state which is associated with poor prognosis [24]. It has been suggested that tumor cells, which are epithelial in origin, may acquire an autocrine or paracrine growth factor-stimulated pathway and thus free themselves from hormonal control [25, 26]. At this point the influence of the ECM compounds may play a crucial role for the further biological behavior of the tumor cells. We have therefore chosen tumor cell lines of varying malignant potential and sex steroid receptor status and analyzed their phenotypical response to different ECM proteins.

We found that attachment to collagen I turned out to be the best growth stimulator for the two well-differentiated cell lines, MCF-7 and T47D. Conversely, in MDA-MB-231 collagen I and the other ECM components did not seem to stimulate proliferation. On one hand, this could indicate that the surface on which they are seeded is not crucial for their growth at all. On the other hand, the results could indicate that due to their high malignant potential, cells grow aggressively and therefore tend to detach from the bottom of the plate.

Laminin displayed the same effect on all three cell lines by inhibiting growth in a monolayer and forcing the cells to form three-dimensional lumps floating into the supernatant. Laminin seemed to interfere with the cell-to-cell contacts. This might explain the small number of attached cells. The reduced adhesion capacity of breast cancer cells to laminin has been previous described [27, 28].

Interestingly, contact with laminin stimulated all three cell lines, especially T47D, to secrete IL-1alpha. It has been reported that laminin reduces estrogen-induced proliferation among MCF-7 and T47D, and therefore seems to promote the development of a hormone-resistant tumor state [29]. ECM assays were performed in the absence of hormones and we found that laminin not only reduced tumor cell numbers or the process of forming dense monolayer cultures, but additionally stimulated cells to release an antimitotic cytokine. There are indications that the loss of estrogen responsiveness in breast cancer might be due to laminin [29]. This might be either due to direct effects of laminin on estrogen receptors or caused indirectly by an increased release of IL-1alpha. It has been reported that IL-1alpha levels correlate inversely with estrogen receptor (ER) levels [30]. Therefore, downregulation of ER triggered by IL-1alpha, might also favor the development of a hormone-resistant tumor state in ER + tumor cells.

Poly-D-lysine induced IL-1alpha-secretion from MDA-MB-231 even more than laminin. MDA-MB-231 released significant amounts of TGF-beta1 when cultured on poly-D-lysine and laminin. Again, these two ECM components were the best inducers of release of IL-1alpha and TGF-beta1 for this malignant cell line.

It is striking that attachment to laminin and poly-D-lysin stimulates secretion of high amounts of IL-1alpha, despite the low number of attached cells. This could be due to the fact that interaction between laminin and cells is a strong inducer of IL-1alpha, however, it might also be possible that floating cells release IL-1alpha into the medium in response to cell-stress. This under- scores our hypothesis that the surface on which cells are cultured has a major impact on their cytokine expression pattern.

TGF-beta1 is known to be a growth inhibiting factor [31, 32]. It is interesting that laminin, the main protein of the basement membrane, stimulates a highly invasive cell line to secrete a growth inhibitor. This autocrine loop seems to attenuate invasive potential, which is underlined by the fact that laminin also leads to IL-1alpha release. Collagen I, IV and fibronectin did not influence MDA-MB-231 in the same way. This might be due to the single steps of invasion and tumor progression. In order to infiltrate the surrounding tissue, tumor cells have to degrade basement membranes. This is controlled by many local factors, such proteases (stromal metalloproteinases), which promote this step [33, 34, 35]. Nevertheless, cytokines such as TGF-beta1 or IL-1alpha might also antagonize

this effect. There seems to be a very delicate balance within the tumor microenvironment between growth inhibiting and promoting factors, leading to reciprocal influences. Before a tumor cell conquers the basement membrane, it is exposed to several effects of each single ECM compound. The sum of all of the positive or negative influences leads to tumor promotion, invasion and metastasis or not. With regard to the highly invasive cell line MDA-MB-231, positive promoting factors probably predominate. However, it is very important to take into consideration that laminin might also be part of a negative paracrine loop within the complex ecosystem, called tumor.

Poly-D-lysine led to a significant release of VEGF by MDA-MB-231, whereas induction of VEGF-release by MCF-7 and T47D was comparatively weak by all other ECM components used. Laminin even seems to have an inhibitory effect on T47D and interestingly on MDA-MB-231, but not on MCF-7. VEGF is a potent angiogenetic factor with a key role in tumor neovascularization. This multistep process involves degradation of the extracellular matrix proteins and activation, proliferation and migration of endothelial cells [36]. Interestingly, collagen IV, but not laminin, stimulates a highly invasive cell line to release VEGF, a positive autocrine loop. Fibronectin and collagen I did not seem to influence VEGF secretion.

Although poly-D-lysine is a synthetic molecule and not part of the ECM-protein skeleton *in vivo*, it is widely used *in vitro* to enhance cell attachment to plastic or glass surfaces. These positively charged polymers are claimed (by the manufacturers) not to stimulate biological activity. However, we observed that all cytokines measured, except bFGF, were released. This should be taken into consideration when poly-D-lysine coating is used in cell cultures.

Laminin and h-fibronectin stimulated T47D to significantly secrete bFGF. Poly-D-lysine seemed to inhibit bFGF release in all three cell lines. This cytokine belongs to a family of angiogenetic factors that are potent mitogens for fibroblasts and endothelial cells and are implicated in epithelial-stromal interactions [37, 38]. Interestingly, the moderate differentiated cell line T47D is stimulated most by laminin and fibronectin to release bFGF.

The reasons for the significant differences in cell kinetics and cytokine expression patterns have not been elucidated so far, but the importance of taking into consideration the fact that tumor cells *in vivo* are part of a complex

network experiencing changes in their microenvironment and responding to these stimuli cannot be stressed enough.

The fact that tumor cell lines of different grade of malignancy exhibit such a diverse cytokine expression pattern in response to similar morphological behavior underlines the importance of cell-matrix interactions. For a long time, only the neoplastic cells were the focus of interest in cancer research, and the stroma was considered a reactive component without major biological and clinical significance. However, it has become clear that the stromal cells and their products (matrix components, growth factors, proteases), condition the phenotype of cancer cells. Thus, tumors represent a complex ecosystem in which interactions between tumor cells, multiple host cells (myofibroblastic, endothelial, and inflammatory cells) and extracellular matrix lead to reciprocal influences resulting in tumor promotion, invasion and metastasis. [39, 40, 41].

A better understanding of the interactions between tumor cells and their microenvironment on the one hand, and each step of malignant transformation on the other, will lead to the development of new therapeutic tools to neutralize the effects of ECM on tumor cells, thereby preventing local invasion and reducing growth promotion.

CONCLUSION

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Figures





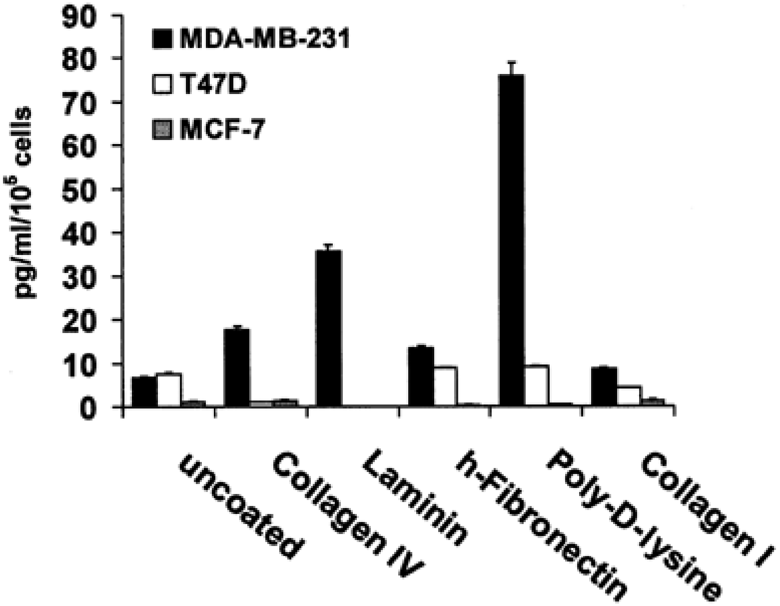

90
80
70
60
50
40
30
20
10
0
pg/ml/10⁵ cells
MDA-MB-231
T47D
MCF-7
uncoated
Collagen IV
Laminin
h-Fibronectin
Poly-D-lysine
Collagen I

Figure 3A

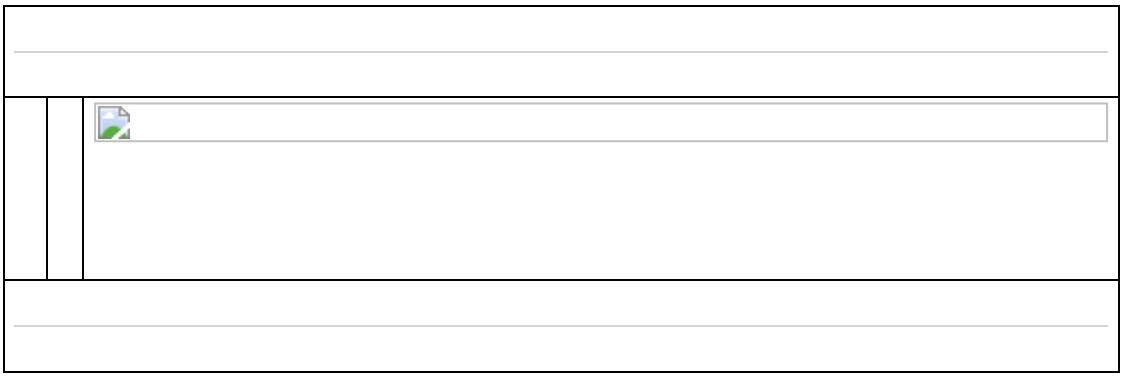
H-TGF-β1 protein secretion in response to ECM components.

MCF-7, T47D and MDA-MB-231 were plated on either untreated culture dishes or on collagen IV, laminin, h-fibronectin, poly-D-lysine or collagen I for 48 hours. H-TGF-β1 was measured in the supernatants by ELISA at day 2. Experiments were performed in triplicate and all values represent the average of protein secretion in pg/ml from duplicate samples. Protein concentrations were normalized to cell numbers and the results are shown in pg/ml/10^5 cells.

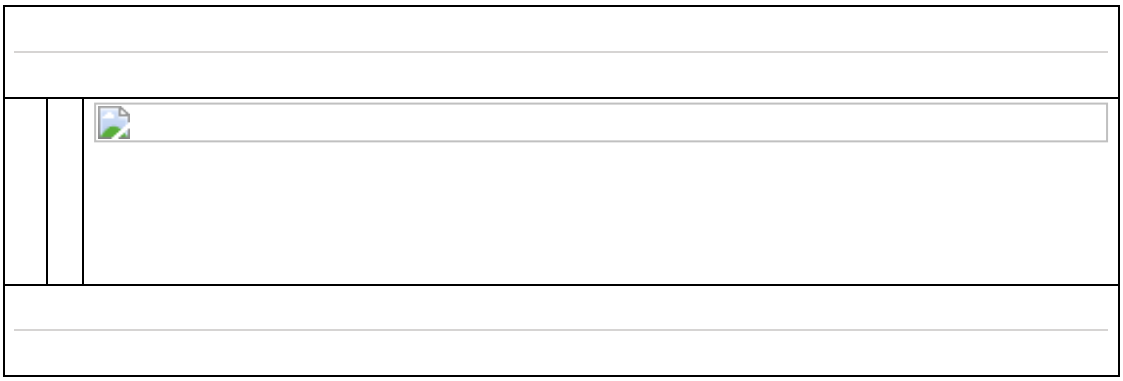












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TGF-beta1, bFGF, VEGF, pulmonary function testing, tracheobronchial aspirate fluid, dexamethasone.

Analysis of the systemic immune response in HIV-1-infected patients suffering from opportunistic Candida infection

C. Punzón, S. Resino, J.-M. Bellón, Ma A. Muñoz-Fernández, M. Fresno
Candida, HIV, cytokine, phagocytes, polymorphonuclear, T cell.

Interferon-alpha suppresses leptin levels: studies in interferon-alpha treated patients with hepatitis C virus infection and murine adipocytes

S. Kaser, A. Kaser, W. Vogel, J. R. Patsch, H. Tilg
interferon-alpha, leptin, chronic hepatitis C virus infection, adipocytes.

The CD14-260 C => T promoter polymorphism co-segregates with the tumor necrosis factor-alpha (TNF-alpha)-308 G => A polymorphism and is associated with the interleukin-1beta (IL-1beta) synthesis capacity of human leukocytes

M. Heesen, B. Bloemeke, B. Bachmann-Mennenga, D. Kunz
CD14, tumor necrosis factor-alpha, interleukin-1beta, endotoxin, genetic polymorphism, linkage disequilibrium, polymerase chain reaction.

Extracellular matrix proteins influence phenotype and cytokine expression in human breast cancer cell lines

K. Hirtenlehne, M. Pec, E. Kubista, C. F. Singer
breast cancer, cell line, extracellular matrix (ECM), tumor cell phenotype, malignant potential, cytokine expression pattern.

Expression and regulation of the macrophage inflammatory protein-1alpha gene by nicotine in rat alveolar macrophages

I.-W. Chong, S.-R. Lin, J.-J. Hwang, M.-S. Huang, T.-H. Wang, J.-Y. Hung, J. D. Paulauskis
nicotine, macrophage inflammatory protein-1alpha (MIP-1alpha), rat alveolar macrophage (RAM).

Recombinant human interleukin (IL)-1beta-mediated regulation of hypoxia-inducible factor-1alpha (HIF-1alpha) stabilization, nuclear translocation and activation requires

an antioxidant/reactive oxygen species (ROS)-sensitive mechanism

J. J. Haddad
antioxidant, airway epithelium, cytokine, HIF-1alpha, IL-1beta, mitochondria, oxygen sensing, reactive oxygen species.

Thioredoxin specifically cross-desensitizes monocytes to MCP-1

S. Pagliei, P. Ghezzi, C. Bizzarri, V. Sabbatini, G. Frascaroli, S. Sozzani, G. Caselli, R. Bertini
thioredoxin, monocytes, MCP-1.

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