IJAE
Vol. 124, n. 1: 42-57, 2019
ITALIAN JOURNAL OF ANATOMY AND EMBRYOLOGY

Research Article - Histology and Cell Biology

Effect of cigarette smoke and treatment with relaxin on guinea pig skin

Angela Silvano*, Silvia Nistri, Laura Calosi, Paolo Romagnoli

Department of Experimental and Clinical Medicine, University of Florence, Italy

Abstract

Cigarette smoking causes microvascular dysfunction and skin aging. Relaxin, primarily but not exclusively involved in reproduction, has connective tissue among its targets. Within a project on the interference of relaxin with the effects of smoke on guinea pigs, we examined the skin response to those stimuli. Adult guinea pigs were exposed to cigarette smoke daily for 8 weeks, and some of them were treated also with relaxin, 1 or 10 μg/die. Controls were treated with relaxin vehicle alone. The skin was analyzed by light and electron microscopy and histochemistry for mast cells and the collagen specific chaperonin Hsp47. The epidermis appeared unaffected by any treatment. In the superficial dermis, smoke led to a decrease in mast cell number and intensity of astra blue staining, suggestive of granule discharge. Relaxin caused further significant reduction in mast cell number. In the superficial and deep dermis, the staining intensity of Hsp47 positive cells, assumed as active fibroblasts, increased upon smoke. The staining intensity decreased gradually in the superficial dermis upon relaxin, reaching significance after treatment with 10 μg/die relaxin, while in the deep dermis it decreased significantly upon treatment with 1 μg/die relaxin and underwent further, significant increase with 10 μg/die relaxin. The results suggest that relaxin can enhance skin mast cell secretory response, possibly antagonizing nicotine induced vasoconstriction and, depending on dose and localization of responding cells, can counteract the profibrotic stimulus of smoke on dermal fibroblasts.

Key words

Hsp47, mast cells, collagen fibres, elastic fibres, skin, relaxin.

Abbreviations

PBS: Phosphate buffered saline
Hsp47: heat shock protein 47
RLX: relaxin
TRITC: trimethylrhodamine.

Introduction

Guinea pig is a useful model for allergic diseases (Morimoto et al., 2014), especially those depending on hypersecretion of factors by mast cell (Ashoori et al., 1996), and for pulmonary damage from inhaled agents (Papi et al., 1999). The skin of guinea

* Corresponding author. E-mail: angela.silvano@hotmail.it

http://www.fupress.com/ijae
DOI: 10.13128/IJAE-25469

pig has been taken as a model to study hypertrophic scars (Aksoy et al., 2002), evaluate the effect of laser treatments on tissue elasticity (Seckel et al., 1997) and test the toxicity of some substances (Korani et al., 2011). It is similar to human skin, however thinner and with Langerhans cells containing fewer Birbeck granules than in humans (Sueki et al., 2000).

Cigarette smoke exerts multiple negative effects on human skin (Yin et al., 2000; Rajagopalan et al., 2016). It affects pathways related to oxidative stress, cell repair and tissue homeostasis, causes microvascular dysfunction (Rossi et al., 2014), induces fibroblast senescence *in vitro* (Yang et al., 2013) and causes premature skin ageing *in vivo* (Morita, 2007); it also negatively affects dendritic cells of the immune system (Nouri-Shirazi and Guinet, 2003).

Relaxin (RLX), initially known for its effects on reproduction and pregnancy, acts on numerous targets including the cardiovascular and respiratory systems and tegument. The availability of human recombinant RLX and the achievements on its biological effects, especially on connective tissue remodelling and cardiovascular physiopathology, have sparkled the interest of clinicians to better explore its therapeutic potential (Bani et al., 2009). RLX has revealed a potent antifibrotic activity which mainly relies on down-regulation of collagen production and of myofibroblast differentiation and increased collagen degradation (Samuel et al., 2007). Its administration has been investigated for the therapy of systemic sclerosis (Samuel et al., 2007; Bennett, 2009) and has been proposed for the treatment of keloids (Lee et al., 2012) . In a swine model, RLX enhanced skin extensibility (Kibblewhite et al., 1992) without significantly affecting dermal thickness (Samuel et al., 2003).

Recent research in our laboratory has investigated the pulmonary and vascular response of guinea pig to cigarette smoke inhalation and the influence of RLX on that response (Pini et al., 2016). Within the framework of that research, we have addressed if smoke stimulates a skin response in guinea pigs and if RLX influences that response.

Material and methods

Reagents

Relaxin (recombinant human H2 RLX, or serelaxin) was kindly provided by the RRCA Relaxin Foundation (Florence, Italy). Kentucky Reference cigarettes 3R4F, each containing 11 mg total particulate matter, 9.4 mg tar and 0.73 mg nicotine, were obtained from the Kentucky Tobacco Research Council (Lexington, KY). Unless otherwise specified, the other reagents used for the experiments were from Sigma-Aldrich (Milan, Italy).

Animals and treatment

Male Hartley albino guinea pigs, average weight 830 g, were bought from Harlan (Correzzana, Italy). Animal handling and use complied with the European Community guidelines for animal care (2010/63/EU) and were approved by the Committee for Animal Care and Experimental Use of the University of Florence. The animals were housed on a 12 h light/dark cycle at 22°C room temperature and had free access to

food and water. The experiments were designed to minimize pain and the number of animals used.

Experimental groups were as follows. (1) Control animals treated with RLX vehicle alone (N = 4). (2) Animals exposed daily to cigarette smoke for 8 weeks (N = 6). (3) Animals exposed daily to cigarette smoke for 8 weeks and treated with RLX 1 μg/d (N = 5). (4) Animals exposed daily to cigarette smoke for 8 weeks and treated with RLX 10 μg/d (N = 4).

The animals were exposed to cigarette smoke in a chamber (2.5 litres), similar to a vacuum desiccator equipped with an open tube for cigarette positioning at one end and a vacuum-connected tube and stopcock at the opposite end, modified from Das et al. (2012). To each group of smoke-exposed animals, five 3R4F reference cigarettes were administered daily. Each cigarette was fitted on the inlet tube and lit; then, a puff of cigarette smoke was introduced into the chamber containing the animals by applying a mild suction (4 cm water for 20 s). The guinea pigs were exposed to the smoke for further 40 s. After a pause of 60 s during which the chamber was opened and ventilated with fresh air, a second puff was administered with the same procedure. The gap between each of the 5 cigarettes/day was 1 h.

Relaxin was dissolved in phosphate buffered saline, pH 7.4 (PBS), and was given by continuous subcutaneous infusion using osmotic minipumps (Alzet, Durect Corporation, Cupertino, CA). The pumps were implanted on the back upon anaesthesia (intraperitoneal ketamine hydrochloride, 100 mg/kg of body weight, and xylazine, 15 mg/kg) one day before starting the exposure to cigarette smoke and were filled with 60 or 600 μg RLX to deliver the daily dose of the drug for the whole duration of the experiment.

At the end of the treatment, the animals were anesthetized as indicated before and sacrificed by decapitation, and tissue samples were collected for analyses.

Light microscopy and histochemistry

Skin samples were fixed in phosphate buffered formaldehyde, pH 7.2 (Immunofix, Bio-Optica, Milan, Italy), for 24 h and embedded in paraffin. Sections about 5 μm thick were stained with haematoxylin and eosin, astra blue (Bani et al., 2006) (Fluka, Buchs, Switzerland), trimethylrhodamine (TRITC)-conjugated avidin (6.25 ng/ml, at 37°C for 1 h) to show mast cell granules (Tharp et al., 1985), picrosirius red to show collagen fibres, Gomori's paraldehyde-fuchsin for elastic fibres. Stained non fluorescent slides were observed under a Microstar IV (Reichert, Depew, NY) or a BA310E microscope (Motic, Hong Kong), both equipped with a T900107 digital photocamera (Tiesselab, Milan, Italy) served by dedicated software (BELView, Bel Engineering Informer Technologies, Madrid, Spain). Fluorescent slides were mounted wet with Fluoromount (Diagnostic BioSystems, Pleasanton, USA), observed in an Axioskop microscope equipped for epifluorescence (Zeiss, Oberkochen, Germany) and captured with an Axio Vision 4 digital system (Zeiss).

To demonstrate the expression of heat shock protein 47 (Hsp47), an intracellular collagen-specific chaperonin (Sluijter et al., 2004), sections were soaked in PBS, followed by citrate buffer (pH 6.0) for 20 min at 95°C to expose antigenic sites and then let to cool to room temperature. Nonspecific binding sites were blocked with 10 ng/mL bovine serum albumin in PBS for 30 min at room temperature, with the addi-

tion of 0.5% triton X-100 (Sigma, Milan, Italy). The primary antibody (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) was applied 1:100 overnight at 4°C; a secondary, goat polyclonal anti-rabbit TRITC-labelled secondary antibody (Sigma-Aldrich; 1:50) was applied for 2 h at room temperature. Omission of primary antibody or substitution with irrelevant ones were used as negative controls. Microscopic observation was as specified above.

Morphometry

In slides stained with picrosirius red and with paraldehyde fuchsin, the intensity of staining per microscopic field was measured with Image J for Windows (NIH, Bethesda, MD) on 6 to 10 microscopic fields per animal at magnification x400. The area of each field was 0,051 mm^2. Each animal was assumed as a sample unit.

The cells labelled with avidin were counted on 20 microscopic fields (magnification x400, field area 0.036 mm^2) of superficial dermis and as many of deep dermis per animal. The cells labelled for Hsp47 were counted on 15-20 microscopic fields per dermal layer and per experimental condition, distributed among all the animals of each group. Each animal was assumed as a sample unit for statistics.

Since the intensity of fluorescent staining of mast cells with TRITC-conjugated avidin was almost always maximal, the amount of substances stored in mast cell granules was evaluated on slides stained with astra blue. Stained cells were delimited by hand, and their section surface area and average stain intensity were measured by Image J (NIH) software; the product of average stain intensity by cell section surface area was assumed as the total amount of label taken up by each cell. The intensity of astra blue staining represents the amount of granules per cell; its decrease indicates degranulation. To evaluate cells immuno-stained for Hsp47, photomicrographs taken at magnification x400 (1388 x 1040 pixel, reproduced tissue surface area 220 x 160 μm) were made binary and the threshold was selected manually so that only labelled cells were visible against the background. The surface area and mean fluorescence intensity of every cell were measured through Image J (NIH) software and multiplied by each other: the product was assumed as the total fluorescence intensity of that cell. For these analyses, each cell was considered a sample unit for statistics.

Electron microscopy

Skin samples were fixed in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, osmicated and embedded in epoxy resin. Sections were stained with gadolinium acetate (Nakakoshi et al., 2011) (Electron Microscopy Sciences, Hatfield, PA) and either lead citrate or bismuth subnitrate, and observed in a Jeol JEM 1010 electron microscope (Tokyo, Japan) at 80 kV. Photomicrographs were taken with a MegaView III digital camera (Soft Imaging System, Muenster, Germany) served by a dedicated software (AnalySIS, Soft Imaging Software, Muenster, Germany).

Statistics

The data are reported as mean ± standard error of the mean (SEM). Differences among groups were evaluated with one-way ANOVA followed by Student–New-

man–Keuls multiple comparison test, using GraphPad Prism 2.0 statistical program (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered significant.

Results

Light microscopy

The skin tissue structure was similar among all groups. The epidermis was thin and with shallow rete pegs, the dermis contained hair follicles, sebaceous glands and a few sweat glands (Fig. 1).

Collagen fibres were thin and interwoven in the superficial dermis, of intermediate thickness in the middle dermis where they were interspersed with adnexa, and arranged in thick bundles in the deep dermis. Quantitative analysis of picrosirius red staining showed no significant differences in the density of collagen fibres among the experimental groups (Fig. 1a-d).

Paraldehyde-fuchsin stained elastic fibres were sparse and thin in the superficial dermis and numerous, relatively thick and interwoven in the middle layer, where they formed a mesh around adnexa. In the deep dermis, elastic fibres were rare and approximately as thick as those in the middle layer (Fig. 1e-h). No significant differences were shown by morphometry among the experimental groups.

Mast cells were significantly more numerous in the superficial dermis than in the intermediate and deep tissue layers. The number of mast cells decreased upon smoking, although not significantly. This number further decreased upon RLX, attaining values significantly lower than controls in the superficial dermis at any RLX dose; this decrease was significantly more marked upon 10 μg/d RLX than upon 1 μg/d RLX (Fig. 2a).

The intensity of mast cell staining with astra blue was significantly reduced by smoking in the superficial dermis. This modification was not significantly affected by RLX at either dosage (Fig. 2b, 3).

Immunohistochemistry

The number of Hsp47 positive cells was slightly higher in the superficial than in the deep dermis. In the deep dermis, it increased upon smoking, an effect that was inhibited by RLX 1 μg/d but not by RLX 10 μg/d (Fig. 2c, 4). However, the differences did not reach significance.

The fluorescence intensity per Hsp47 labelled cell in control animals was higher, albeit not significantly, for the deep than the superficial dermis; upon smoking the fluorescence intensity per cell increased significantly in both dermal layers. RLX counteracted in part this increase, with different dose-response relationship between dermal layers. In the superficial dermis the inhibition was progressively more marked with the dose and fluorescence values significantly lower than controls were attained with 10 μg/d. In the deep dermis the increase was significantly inhibited already by RLX 1 μg/d while RLX 10 μg/d produced a significant increase of fluorescence intensity over that upon smoke (Fig. 2d, 4).

Hsp47 positive cells were larger in the deep than in the superficial dermis in all experimental conditions, but the difference was not significant in control conditions.

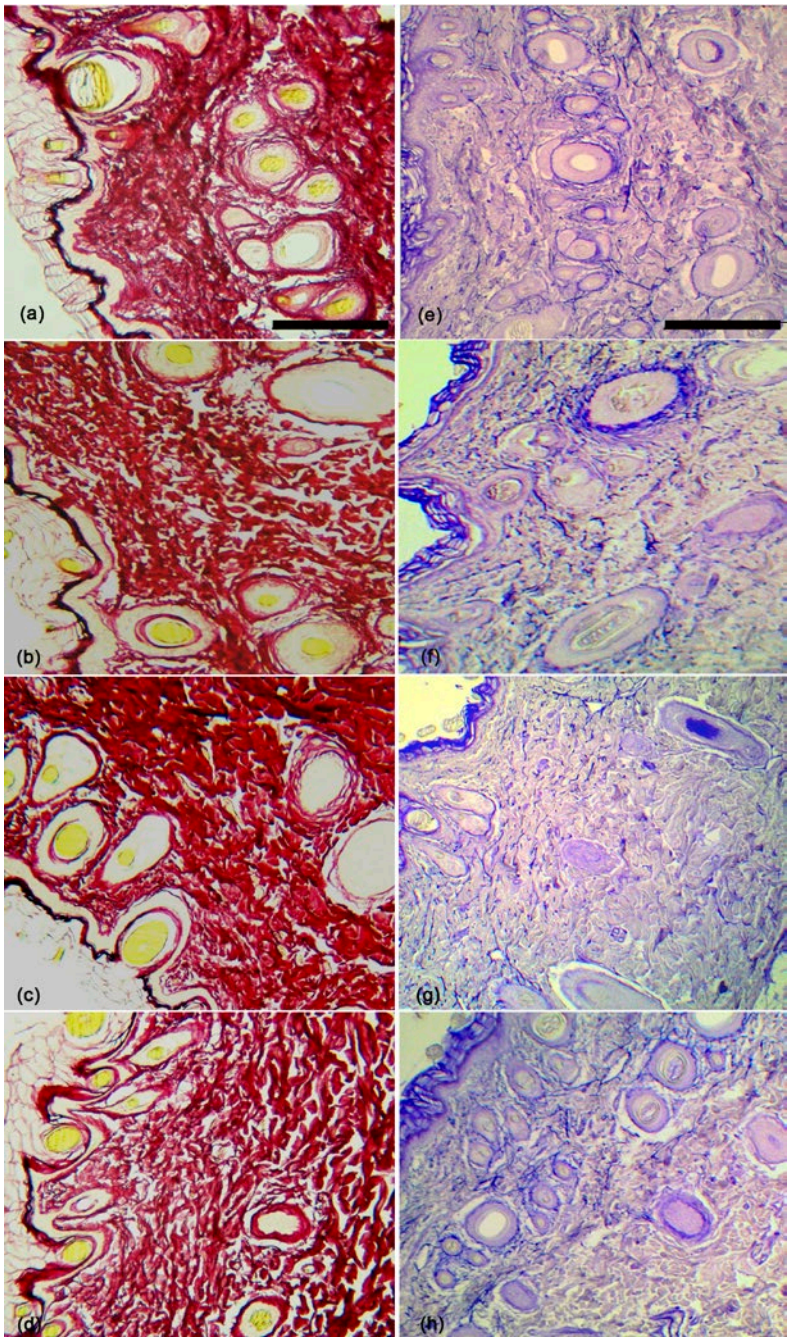

Figure 1. Guinea pig control skin stained with: (a-d) picrosirius red for collagen fibres; (e-h) paraldehyde fuchsin for elastic fibres. (a-e) Control; (b-f) Smoke; (c-g) Smoke plus RLX 1µg/d; (d-h) Smoke plus RLX 10µg/d. Scale bar = 170 µm.

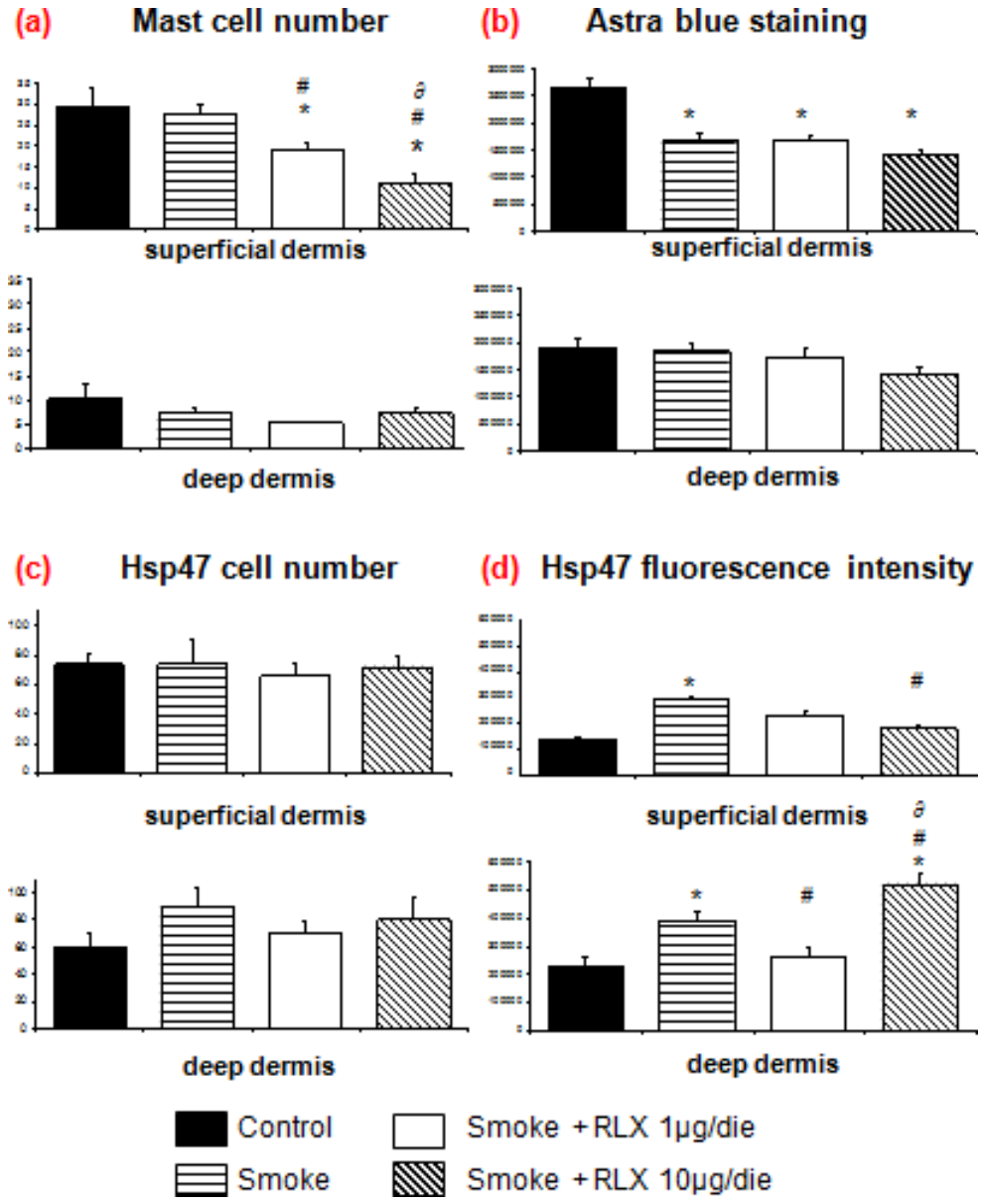

Figure 2. Cell counts and staining intensity. (a) Mast cell number per mm2 of skin section surface area. (b) Astra blue staining intensity of mast cells, in arbitrary units. (c) Hsp47 positive cell number per mm2 of skin section surface area. (d) Fluorescence intensity of Hsp47 positive cells, in arbitrary units. RLX: relaxin (at the indicated dose in µg/d). Symbols indicate significance ($p<0.05$) between experimental conditions, within a same layer: (*) compared with control; (#) compared with smoke; (∂) compared with RLX 1µg/d. For mast cell number and astra blue staining, control values in the deep dermis were significantly different ($p<0.05$) from those in the superficial dermis.

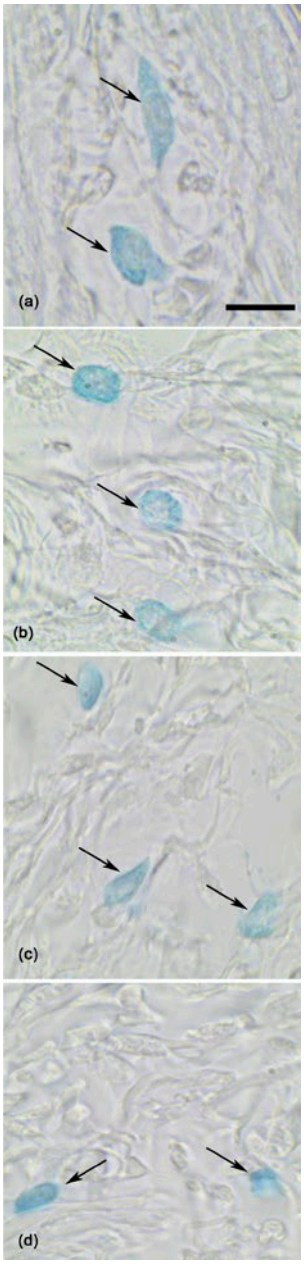

Figure 3. Astra blue stain of mast cells. Example of staining for the superficial dermis (a-d). a: Control. b: Smoke. c: Smoke plus relaxin 1µg/d. d: Smoke plus relaxin 10 µg/d. Scale bar = 20 µm.

Significant differences were induced by treatment in the deep dermis: smoke led to cell enlargement over controls, that was only slightly inhibited by RLX 1 μg/d (not significant) and on the contrary markedly increased upon RLX 10 μg/d ($p<0.05$ versus control and RLX 1 μg/d) (Fig. 5).

Electron microscopy

The epidermis appeared thin, with one layer of basal cells, one or two layers of prickle cells and one layer of granular cells, topped by a few layers of horny cells. Langerhans cells were located between the basal and prickle cell layers, had a small, ovoid body and few Birbeck granules with lightly marked inner structure; some Birbeck granules were bent, some had at one end a pale, tubular or slightly dilated portion (Fig. 6).

The dermis contained few cells of different types: fibroblasts, macrophages with well developed lysosomes, and mast cells with granules having a reticulated inner structure. In the basement membrane, the reticular layer lying under the lamina densa was thin and poor in fibrils. Blood vessels and fine nerves with non-myelinated fibres were identified in the superficial dermis; a few myelinated nerve fibres were found in nerves further away from the epidermis; the dermis also contained arrectores pili muscles.

The electron microscopic structure of the epidermis and dermis did not appear affected by any treatment.

Discussion

Normal guinea pig skin

The findings on the normal guinea pig skin were coherent with those reported by Sueki et al. (2000). It is possible that epidermal Langerhans cells have fewer Birbeck granules than those in human epidermis, because the guinea pig skin is protected by a fur, as suggested by data from hairless rat (Itagaki et al., 1995).

In the dermis, mast cells were more numerous in the superficial than in the deep part of the tissue, conceivably in relation with the different density of blood microvessels to which mast cells are associated. Many Hsp47 positive cells were seen sparse in the dermis. Hsp47 can be assumed as a marker of active fibroblasts

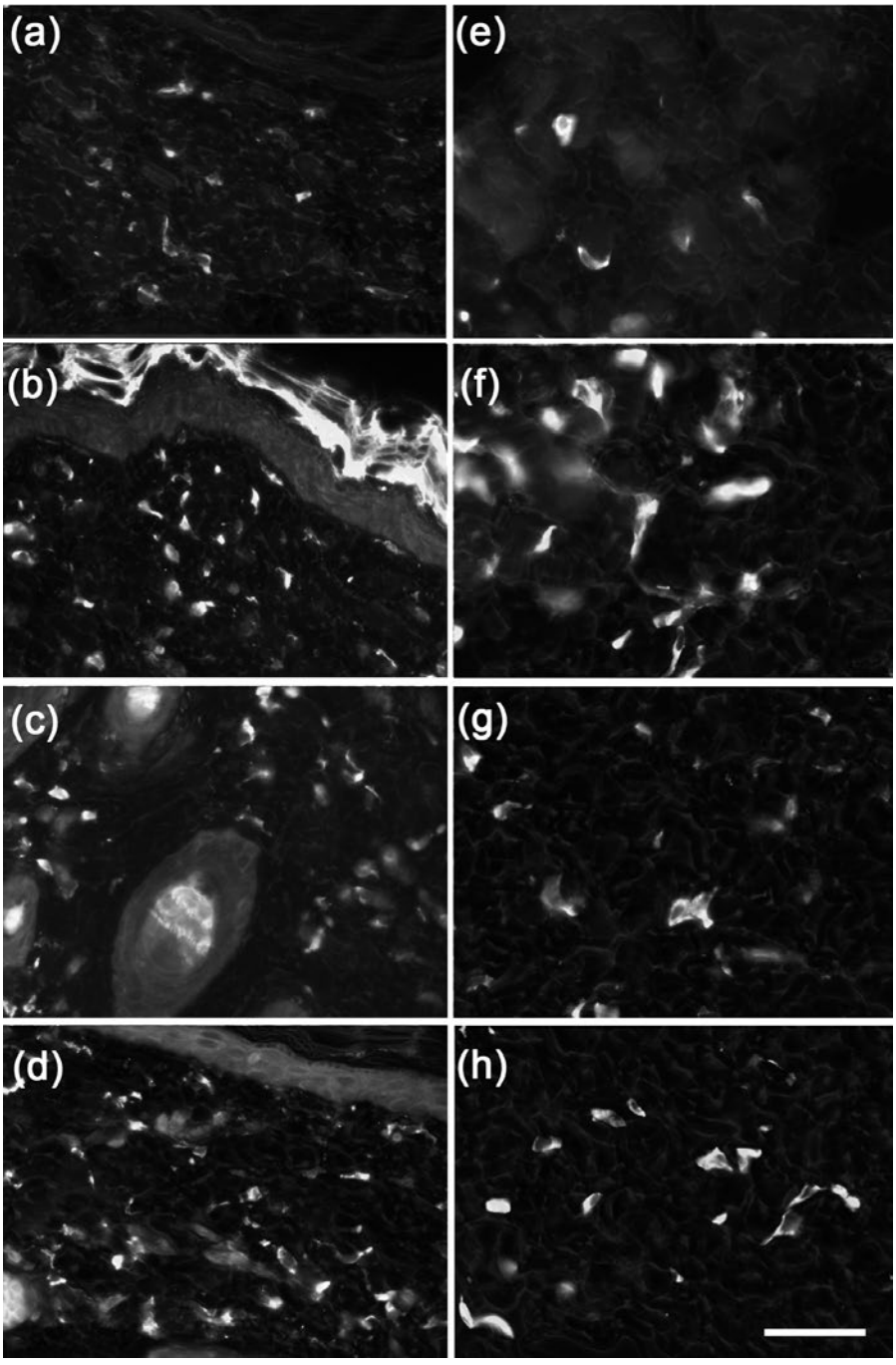

Figure 4. Hsp47 positive cells in superficial dermis (a-d) and deep dermis (e-h). a, e: Control. b, f: Smoke. c, g: Smoke plus relaxin 1µg/d. d, h: Smoke plus relaxin 10 µg/d. Scale bar = 40 µm.

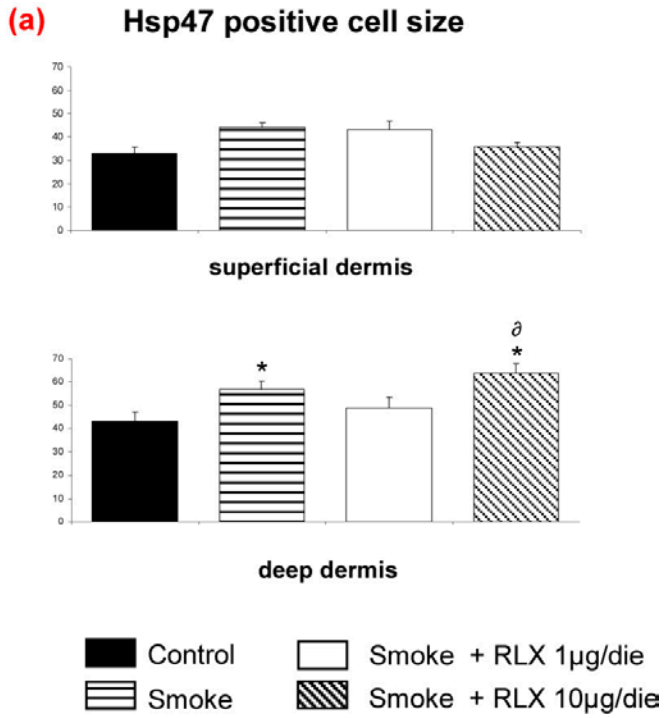

Figure 5. Size of Hsp47 positive cells in µm2. In the deep layer Hsp47 positive cells have a larger size than the Hsp47 positive cells in the superficial dermis in all experimental conditions. Symbols indicate significance ($p<0.05$) between experimental conditions, within a same layer: (*) compared with control; (∂) compared with relaxin 1µg/d. The size of cells in the deep dermis of the animals exposed to cigarette smoke and those exposed to smoke plus relaxin 10 µg/d was significantly larger than that of the cells of superficial dermis in the same conditions.

(Mehta et al., 2005). This chaperonin does not seem to have been an object of study in the guinea pig skin until now.

Effects of smoke on guinea pig skin

Smoke did not lead to changes in the epidermis, while it appeared to affect the dermis. Mast cells underwent reduction in number and in the intensity of astra blue staining, which depends on the amount of cytoplasmic granules. Both these parameters indicate active secretion of mast cell granules, because highly degranulated cells are no more recognizable histologically, and hint that mast cell secretion was increased by smoking. Components of cigarette smoke, such as nicotine, cause vasoconstriction of microvessels in the skin, which may lead to decreased moisture content and dryness, and poor wound healing (Mosely et al., 1977; Rossi et al., 2014). Increased mast cell degranulation in the superficial dermis may represent an effort to counteract vasoconstriction in the superficial dermis.

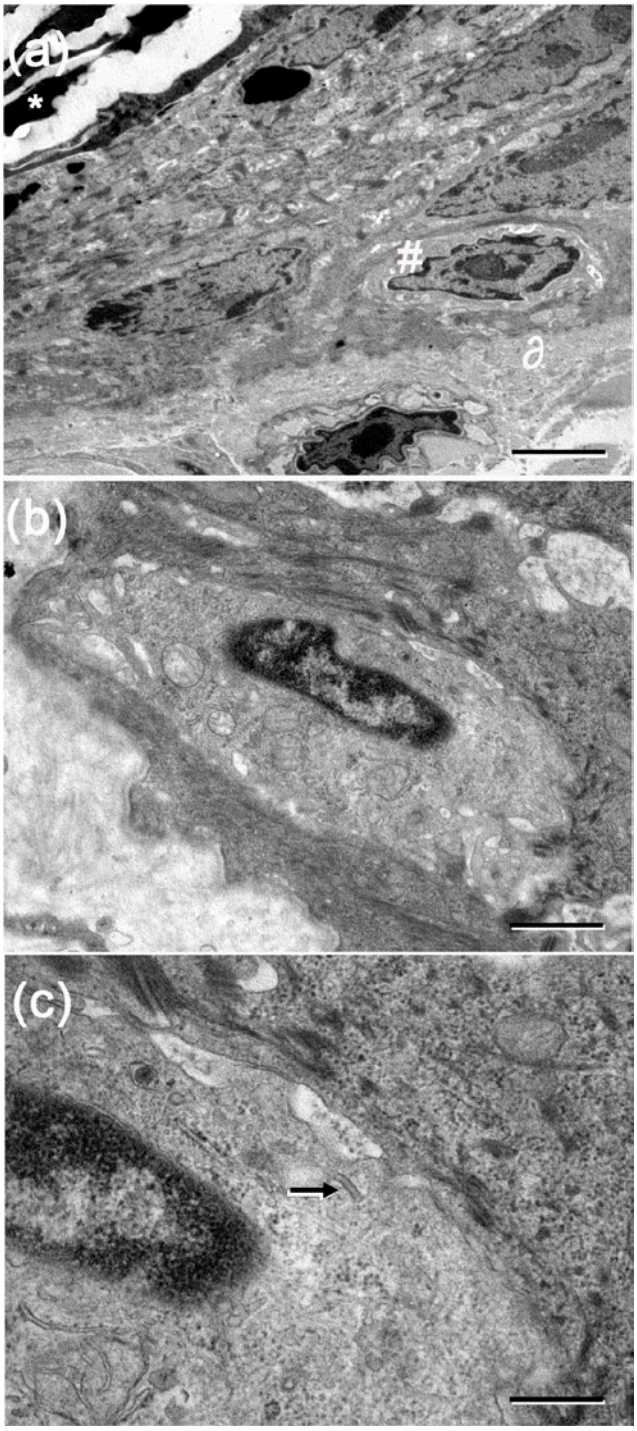

Figure 6. Electron microscopy. (a): Overview of epidermis from a smoke exposed guinea pig. The horny layer is at the upper left corner. The asterisk (*) indicates the granular layer. The hashtag (#) indicates a Langerhans cell. ∂ indicated the dermis. Scale bar = 2 µm. (b): Higher magnification of a Langerhans cell. Scale bar = 1 µm. (c): Detail of the cell of panel (b), showing a small Birbeck granule (arrow). Scale bar = 500 nm.

Smoke led to increase in the staining intensity of Hsp47 positive cells, assumed as active fibroblasts, at all dermal levels. The increase in production of this chaperonin was paralleled by an increase in cell size, especially in the deep dermis. *In vitro* studies had shown that skin fibroblasts undergo widespread damage if exposed to cigarette smoke extract, with inhibition of cell viability and proliferation (Rossi et al., 2014). The present results suggest that skin fibroblasts are not damaged by smoke *in vivo*, at least in this guinea pig model, at variance with smoke extract effect *in vitro*: this discrepancy may depend on differences in composition and concentration of smoke-derived substances that reach the tissue through the bloodstream upon smoke inhalation. Smoke tar does not reach the skin while carbon monoxide and nicotine do. This may affect cells both directly (through a receptor mediated mechanism) and indirectly (through vasoconstriction and damage to microcirculation). Fibroblasts express the α7-nicotinic receptor in humans and the α3-nicotinic receptor in mice (Arredondo, 2003). The stimulation of nicotinic receptor *in vitro* and *ex vivo* leads to reduced proliferation of fibroblasts (Xanthoulea et al., 2013). Studies *in vitro* have shown alternatively an increase (Chamson et al., 1992) or a decrease in collagen production by fibroblasts upon treatment with tobacco smoke extracts (Yin et al., 2000). *In vivo* the effect of tobacco smoke extract on mouse skin depends on the way of administration: at variance with topical or intradermal treatment, systemic (intraperitoneal) administration seems to have no effect on skin collagen bundles (Yang et al., 2013). These discrepancies give strength to *in vivo* animal models as suitable tools to study what may happen in clinics.

The effects of smoking on guinea pig skin are partially counteracted by RLX.

Our study indicates a partial protective effect of RLX on skin injury in the animals exposed to cigarette smoke, because the further reduction in number of dermal mast cells induced by RLX upon cigarette smoke exposure may be assumed as a sign of degranulation, hence of enhanced secretion of vasodilator and vasoprotective agents by these cells. In other organs RLX has an opposite effect on mast cells than that observed in the present study on the skin: in fact, it inhibits peritoneal and heart mast cell degranulation and histamine release *in vivo* (Masini et al., 1994; Nistri et al., 2008). These opposite effect of RLX could be related to heterogeneity of mast cells settled in different anatomical sites and tissues, as described in the literature (Dwyer et al., 2016). Therefore. RLX may be partially protective against cigarette smoke effects on the skin, but not on visceral organs.

Relaxin counteracted the increase in the staining intensity of Hsp47 positive fibroblasts in the superficial dermis in a dose dependent manner, RLX at low dose also counteracted such an increase in the deep dermis whereas at the latter level the higher dose of RLX stimulated a paradoxical increase in Hsp47 staining intensity over control values. This suggests a down-regulation of collagen production by low dose RLX, which fits well with its well-known anti-fibrotic properties. It can be speculated that the inconspicuous differences found in dermal collagen fibres depend on the fact that 8 weeks were insufficient to cause extensive remodelling in the skin tissue not directly exposed to the noxious effects of smoke, assuming that major modifications of collagen fibres usually take place in the long time. The paradoxical effect of the higher RLX dose may be explained by RLX receptor down-regulation by excess

ligand because of dimerization and negative cooperativity between receptor molecules (Svendsen et al., 2009). The difference between the superficial and the deep dermis in the fibroblast response to RLX administration upon cigarette smoke hints to a tissue specificity in the response to RLX, which needs to be taken into consideration when planning to use this molecule in a clinical setting.

Relaxin has not proven effective in the clinics to reverse established alterations of systemic sclerosis (Khanna et al., 2009), despite relaxin is able to interfere with mechanisms of fibrosis in several organs (McVicker and Bennett, 2017; Samuel et al., 2017). Those data and the present results suggest that an influence of possible clinical relevance can be expected during the initial, dynamic phase of any fibrotic process (be it scarring, systemic sclerosis or else), before fibrosis has established.

In conclusion, smoking leads to an increase in fibroblast activity in the guinea pig skin, as indicated by Hsp47 enhanced expression, and RLX can counteract this effect depending on the dose and the localization of responding cells. Guinea pig mast cells undergo modifications suggesting secretion of their granules after smoke and even more upon administration of RLX, which may represent a protective reaction against the vasoconstrictor effect of nicotine. This study also showed that guinea pig fibroblasts and mast cells behave differently between the superficial and the deep dermis. The presence of histological and functional compartments in the skin should be taken into account when planning and evaluating experiments and clinical treatments on this tissue in guinea pigs and possibly in other species including humans.

Acknowledgements

The authors gratefully acknowledge Prof. D. Bani (University of Florence) for discussion of the experimental design and the manuscript, Mr. D. Guasti for assistance in electron microscopy, and University of Florence, Ente Cassa di Risparmio di Firenze and Foemina Foundation for financial support.

Conflict of interest

The senior author is Editor-in Chief of the Italian Journal of Anatomy and Embryology.

References

Aksoy M.H., Vargel I., Canter I.H., Erk Y., Sargon M., Pinar A., Tezel G.G. (2002) A new experimental hypertrophic scar model in guinea pigs. Aesthetic Plast. Surg. 26: 388-396.

Arredondo J., Hall L.L., Ndoye A., Nguyen V.T., Chernyavsky A.I., Bercovich D., Orr-Urtreger A., Beaudet A.L., Grando S.A. (2003) Central role of fibroblast alpha3 nicotinic acetylcholine receptor in mediating cutaneous effects of nicotine. Lab. Invest. 83: 207-225.

Ashoori F., Suzuki S., Zhou J., Nishigaki I., Takahashi R. (1996) Possible contributions of mastocytosis, apoptosis, and hydrolysis in pathophysiology of randomized skin flaps in humans and guinea pigs. Plast. Reconstr. Surg. 98: 491-501.

Bani D., Giannini L., Ciampa A., Masini E., Suzuki Y., Menegazzi M., Nistri S., Suzuki H. (2006) Epigallocatechin-3-gallate reduces allergen-induced asthma-like reaction in sensitized guinea pigs. J. Pharmacol. Exp. Ther. 317: 1002-1111.

Bani D., Yue S.K., Bigazzi M. (2009) Clinical profile of relaxin, a possible new drug for human use. Curr. Drug. Saf. 4: 238-249.

Bennett R.G. (2009) Relaxin and its role in the development and treatment of fibrosis. Transl. Res. 154: 1-6.

Chamson A., Frey J., Hivert M. (1982) Effects of tobacco smoke extracts on collagen biosynthesis by fibroblast cell cultures. J. Toxicol. Environ. Health 9: 921-932.

Das A., Dey N., Ghosh A., Das S., Chattopadhyay D.J., Chatterjee I.B. (2012) Molecular and cellular mechanisms of cigarette smoke-induced myocardial injury: prevention by vitamin C. PLoS One 7: e44151 [13 pages].

Dwyer D.F., Barrett N.A., Austen K.F.; Immunological Genome Project Consortium (2016) Expression profiling of constitutive mast cells reveals a unique identity within the immune system. Nat. Immunol. 17: 878-887.

Itagaki S., Ishii Y., Lee M.J., Doi K. (1995) Dermal histology of hairless rat derived from Wistar strain. Exp. Anim. 44: 279-284.

Khanna D., Clements P.J., Furst D.E., Korn J.H., Ellman M., Rothfield N., Wigley F.M., Moreland L.W., Silver R., Kim Y.H., Steen V.D., Firestein G.S., Kavanaugh A.F., Weisman M., Mayes M.D., Collier D., Csuka M.E., Simms R., Merkel P.A., Medsger T.A. Jr, Sanders M.E., Maranian P., Seibold J.R.; Relaxin Investigators and the Scleroderma Clinical Trials Consortium. (2009) Recombinant human relaxin in the treatment of systemic sclerosis with diffuse cutaneous involvement: a randomized, double-blind, placebo-controlled trial. Arthritis Rheum. 60, 1102-1111.

Kibblewhite D., Larrabee W.F. Jr., Sutton D. (1992) The effect of relaxin on tissue expansion. Arch. Otolaryngol. Head. Neck. Surg. 118: 153-156.

Korani M., Rezayat S.M., Gilani K., Arbabi Bidgoli S., Adeli S. (2011) Acute and subchronic dermal toxicity of nanosilver in guinea pig. Int. J. Nanomedicine 6: 855-862.

Lee W.J., Choi I.K., Lee J.H., Lee J.S., Kim Y.O., Rah D.K., Yun C.O. (2012) Relaxin-expressing adenovirus decreases collagen synthesis and up-regulates matrix metalloproteinase expression in keloid fibroblasts: in vitro experiments. Plast. Reconstr. Surg. 130: 407e-417e.

Masini E., Bani D., Bigazzi M., Mannaioni P.F., Sacchi-Bani T. (1994) Effects of relaxin on mast cells. In vitro and in vivo studies in rats and guinea pigs. J. Clin. Invest. 94: 1974-1980.

McVicker B.L., Bennett R.G. (2017) Novel anti-fibrotic therapies. Front. Pharmacol. 8: 318 [21 pages].

Mehta TA., Greenman J., Ettelaie C., Venkatasubramaniam A., Chetter IC., McCollum PT. (2005) Heat shock proteins in vascular disease a review. Eur. J. Vasc. Endovasc. Surg. 29: 395-402.

Morimoto T., Higaki T., Ota M., Inawaka K., Kawamura S., Bungo T. (2014) Effects of simultaneous exposure to mixture of two skin sensitizers on skin sensitization response in guinea pig and mice. J. Toxicol. Sci. 39: 163-171.

Morita A. (2007) Tobacco smoke causes premature skin aging. Dermatol Sci 48: 169-175.

Mosely L.H., Finseth F. (1977) Cigarette smoking: impairment of digital blood flow and wound healing in the hand. Hand 9: 97-101.

Nakakoshi M., Nishioka H., Katayama E. (2011) New versatile staining reagents for biological transmission electron microscopy that substitute for uranyl acetate. J. Electron. Microsc. 60: 401-407.

Nistri S., Cinci L., Perna A.M., Masini E., Bani D. (2008) Mast cell inhibition and reduced ventricular arrhythmias in a swine model of acute myocardial infarction upon therapeutic administration of relaxin. Inflamm. Res. 57 (Suppl. 1): S7-S8.

Nouri-Shirazi M., Guinet E. (2003) Evidence for the immunosuppressive role of nicotine on human dendritic cell functions. Immunology 109: 365-373.

Papi A., Amadesi S., Chitano P., Boschetto P., Ciaccia A., Geppetti P., Fabbri L.M., Mapp C.E. (1999) Bronchopulmonary inflammation and airway smooth muscle hyperresponsiveness induced by nitrogen dioxide in guinea pigs. Eur. J. Pharmacol. 374: 241-247.

Pini A., Boccalini G., Lucarini L., Catarinicchia S., Guasti D., Masini E., Bani D., Nistri S. (2016) Protection from cigarette smoke-induced lung dysfunction and damage by H2 relaxin (serelaxin). J. Pharmacol. Exp. Ther. 357: 451-458.

Rajagopalan P., Nanjappa V., Raja R., Jain A.P., Mangalaparthi K.K., Sathe G.J., Babu N., Patel K., Cavusoglu N., Soeur J., Pandey A., Roy N., Breton L., Chatterjee A., Misra N., Gowda H. (2016) How does chronic cigarette smoke exposure affect human skin? A global proteomics study in primary human keratinocytes. OMICS 20: 615-626.

Riganò R., Profumo E., Buttari B., Tagliani A., Petrone L., D'Amati G., Ippoliti F., Capoano R., Fumagalli L., Salvati B., Businaro R. (2007) Heat shock proteins and autoimmunity in patients with carotid atherosclerosis. Ann. N Y. Acad. Sci. 1107: 1-10.

Rossi M., Pistelli F., Pesce M., Aquilini F., Franzoni F., Santoro G., Carrozzi L. (2014) Impact of long-term exposure to cigarette smoking on skin microvascular function. Microvasc. Res. 93: 46-51.

Samuel C.S., Sakai L.Y., Amento E.P. (2003) Relaxin regulates fibrillin 2, but not fibrillin 1, mRNA and protein expression by human dermal fibroblasts and murine fetal skin. Arch. Biochem. Biophys. 411: 47-55.

Samuel C.S., Lekgabe E.D., Mookerjee I. (2007) The effects of relaxin on extracellular matrix remodeling in health and fibrotic disease. Adv. Exp. Med. Biol. 612: 88-103.

Samuel C.S., Royce S.G., Hewitson T.D., Denton K.M., Cooney T.E., Bennett R.G. (2017) Anti-fibrotic actions of relaxin. Br. J. Pharmacol. 174: 962-976.

Seckel B., Younai S., Wang K. (1997) Skin tightening effects of the ultrapulse CO_2 laser. Plast. Reconstr. Surg. 102: 872-877.

Sluijter J.P.G., Smeets M.B., Velema E., Pasterkamp G., de Kleijn D.P.V. (2004) Increased collagen turnover is only partly associated with collagen fiber deposition in the arterial response to injury. Cardiovasc. Res. 61: 186-195.

Sueki H., Gammal C., Kudoh K., Kligman A.M. (2000) Hairless guinea pig skin: anatomical basis for studies of cutaneous biology. Eur. J. Dermatol. 10: 357-364.

Svendsen A.M., Vrecl M., Knudsen L., Heding A., Wade J.D., Bathgate R.A., De Meyts P., Nøhr J. (2009) Dimerization and negative cooperativity in the relaxin family peptide receptors. Ann. N Y. Acad. Sci. 1160: 54-59.

Tharp M.D., Seelig L., Tigellar R., Bergstresser P.R. (1985) Conjugated avidin binds to mast cells granules. J. Histochem. Cytochem. 33: 27-32.

Xanthoulea S., Deliaert A., Romano A., Rensen S.S., Buurman W.A., van der Hulst R.R. (2013) Nicotine effect on inflammatory and growth factor responses in murine cutaneous wound healing. Int. Immunopharmacol. 17: 1155-1164.

Yang G.Y., Zhang C.L., Liu X.C., Qian G., Deng D.Q., Liu X.C. (2013) Effects of cigarette smoke extracts on the growth and senescence of skin fibroblasts in vitro. Int. J. Biol. Sci. 9: 613-623.

Yin L., Morita A., Tsuji T. (2000) Alterations of extracellular matrix induced by tobacco smoke extract. Arch. Dermatol. Res 292: 188-194.