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Alterations of extracellular matrix induced by tobacco smoke extract

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Abstract **Epidemiologic studies have indicated the association between tobacco smoking and skin aging, but the exact mechanism of tobacco smoke-induced premature skin aging is currently unknown. In this study, we investigated the alterations of collagen, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in human fibroblasts treated with tobacco smoke extract. Human fibroblasts were exposed to different concentrations of water-soluble extract from tobacco smoke. Human fibroblasts irradiated with ultraviolet A1 (UVA1) were used as positive controls because the mechanism of UVA1-mediated MMP expression has been well characterized. The expression of MMP and TIMP was analyzed semiquantitatively following reverse transcriptase-polymerase chain reaction. Production of type I and type III collagens was detected by Western blotting and biosynthesis of new collagen was assessed by ^{3}H-proline incorporation. Upon treatment with tobacco smoke extract or UVA1 irradiation, the expression of MMP-1 and MMP-3 mRNA was significantly increased in a dose-dependent manner. Maximum induction was observed with 25 μl/ml tobacco smoke extract. In contrast, the expression of TIMP-1 and TIMP-3 mRNA remained unchanged. Western blotting of the supernatant revealed that type I and type III collagens were decreased as compared with untreated controls. Collagen biosynthesis was significantly reduced by 40.1% following treatment with 25 μl/ml tobacco smoke extract. Sodium azide, L-ascorbic acid and Trolox (a water-soluble vitamin E) prevented both the UVA1- and the tobacco-induced alteration of MMP-1. These observations suggest that the imbalance of connective tissue matrix components might contribute to the molecular basis for premature skin aging in smokers. They also suggest that reactive oxygen species including singlet oxygen mediate this process.**

Key words Matrix metalloproteinase · Extracellular matrix · Reactive oxygen species

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Introduction

Tobacco smoking is a health hazard and each year is responsible for more than 3 million deaths worldwide (Smith and Fenske 1996). It is well known that tobacco smoking is strongly associated with lung cancer, emphysema, chronic bronchitis, cardiovascular disease and other serious internal diseases and cancers (Bartsch et al. 1993; Penn et al. 1994; Schafer et al. 1997; Sonnenfeld and Hudgens 1983; Stefanadis et al. 1997).

Tobacco smoking also has deleterious effects on the skin and "smoker's wrinkles" are a typical clinical feature of smokers (Daniell 1971; Ernster et al. 1995; Frances 1992; Grady and Ernster 1992; Kadunce et al. 1991; Kottke 1991; Lange and Schnohr 1994). A recent epidemiologic study has clearly shown that tobacco smoking is one of the numerous factors contributing to premature skin aging, which is independent of age, sex, pigmentation, sun exposure history, alcohol consumption and other factors (Kadunce et al. 1991). Little is known, however, about the molecular and cellular mechanisms underlying this deleterious effect.

Skin aging is a complex biologic process which includes intrinsic and extrinsic aging. Intrinsic aging affects the skin in a manner similar to other organs. Superimposed on this process, environmental factors such as tobacco smoke and ultraviolet (UV) radiation contribute to extrinsic aging. Normal human skin is dependent on the balance between the biosynthesis and degradation of extracellular matrix (ECM). Matrix metalloproteinases (MMPs) play a key role in ECM remolding in the skin.

MMP-1 (interstitial collagenase) degrades extracellular fibers comprised of type I and type III collagens. MMP-3 (stromelysin-1) degrades proteoglycans and fibronectin as well as native type III collagen (Giambernardi et al. 1998; Kuroda and Shinkai 1997). The activity of MMPs is specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs). Any imbalance between the matrix components and their degrading factors could potentially lead to abnormal accumulation of ECM, resulting in loss of recoil capacity and tensile strength with wrinkle formation (Uitto and Bernstein 1998). We have recently found that tobacco smoke extract induces wrinkle formation in experimental animals (Yin et al., manuscript in preparation).

To determine the molecular mechanism of tobacco smoke extract-induced premature skin aging, we examined the production and biosynthesis of collagen as well as the expression of MMPs and TIMPs in cultured human fibroblasts upon treatment with tobacco smoke extract or UVA1 radiation.

Materials and methods

Skin fibroblasts and cell culture

Skin fibroblasts from three healthy individuals who were nonsmokers (41-year-old male, 75-year-old female, and 73-year-old female) were separated from tissue explant cultures which were trimmed of excess fat and subcutaneous tissue and minced into 2–3-mm fragments. Subsequently, cDMEM (complete Dulbecco's modified Eagle's medium) was added, and the explants were incubated at 37 °C in an atmosphere containing 5% CO_2. Fibroblasts at passages 3 to 18 were used. cDMEM comprised DMEM (Sigma Chemical Co, St. Louis, Mo.), 10% fetal calf serum (FCS; BioSciences PTY, Australia), 100 units/ml antibiotic-antimycotic, 10 m*M* MEM nonessential amino acid solution, 200 m*M* L-glutamine and HEPES buffer (Sigma).

Preparation and treatment of tobacco smoke extract

At room temperature, aqueous smoke solution (tobacco smoke extract) was prepared by passing the smoke from a cigarette through phosphate-buffered saline (PBS; Sigma). Two round-bottomed flasks containing 20 ml PBS were used in this system. The side of one flask (A) was connected to the pump, the other (B) to a cigarette. One cigarette (with paper and filter) was pumped for 2 s at 1-min intervals. The smoke solution from two flasks (A + B = 40 ml) was collected and then adjusted to pH 7.4 with sodium bicarbonate water and filtered through a 0.22-μm filter (Millipore Co., Bedford, Mass.) (Stuart et al. 1978). For tobacco smoke extract stimulation, cells at near-confluence were changed to starvation medium (without FCS) for 2 h prior to incubation with the designated concentrations of tobacco smoke extract for 24 h. Subsequently, the medium and cell layer were collected for the further studies. No cytotoxic effect was found up to 25 μl/ml.

UV irradiation

For UVA1 irradiation, the medium was replaced by PBS. Lids were removed and cells were exposed to UVA1 radiation (10, 20, 30 J/cm^2) from a UVA1 Sellamed System Dr Sellmeier (Sellas, Gevolsburg, Germany) as previously described (Morita et al. 1997).

RNA extraction

The total RNA was extracted using a modified guanidinium isothiocyanate method (Chamberlain and Burgoyne 1996) using an Rneasy minikit (Qiagen, Hilden, Germany). The amount of RNA was estimated using a GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech, Uppsala, Sweden) and then 1 μg RNA was routinely taken for RT-PCR assay.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

For cDNA synthesis, 1 μg RNA, 5 μl bulk first-strand reaction mix (containing murine reverse transcriptase), 1 μl of a pd $(N)_6$ random hexamer primer and 1 μl dithiothreitol solution (Amersham Pharmacia Biotech) were incubated at 65 °C for 10 min in a final volume of 15 μl. To ensure that similar amounts of cDNA were used for PCR, samples were assessed for expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The cDNA was amplified in the presence of 10 units Taq polymerase (Amersham Pharmacia Biotech) per microgram cDNA and 10 pmol of both the sense and antisense primer or GAPDH oligonucleotides in PCR buffer (50 m*M* KCl, 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl). PCR was conducted with 25 cycles of amplification, which was within the linear amplification range for all cDNAs (denaturation for 1 min at 95 °C, annealing for 1 min at 60 °C, and extension for 2 min at 72 °C). PCR products (18 μl) were electrophoresed in 1% agarose gel and visualized using ethidium bromide. Fragments of the expected sizes were obtained (600 bp for GAPDH, 479 bp for MMP-1, 313 bp for MMP-3, 517 bp for TIMP-1, and 322 bp for TIMP-3).

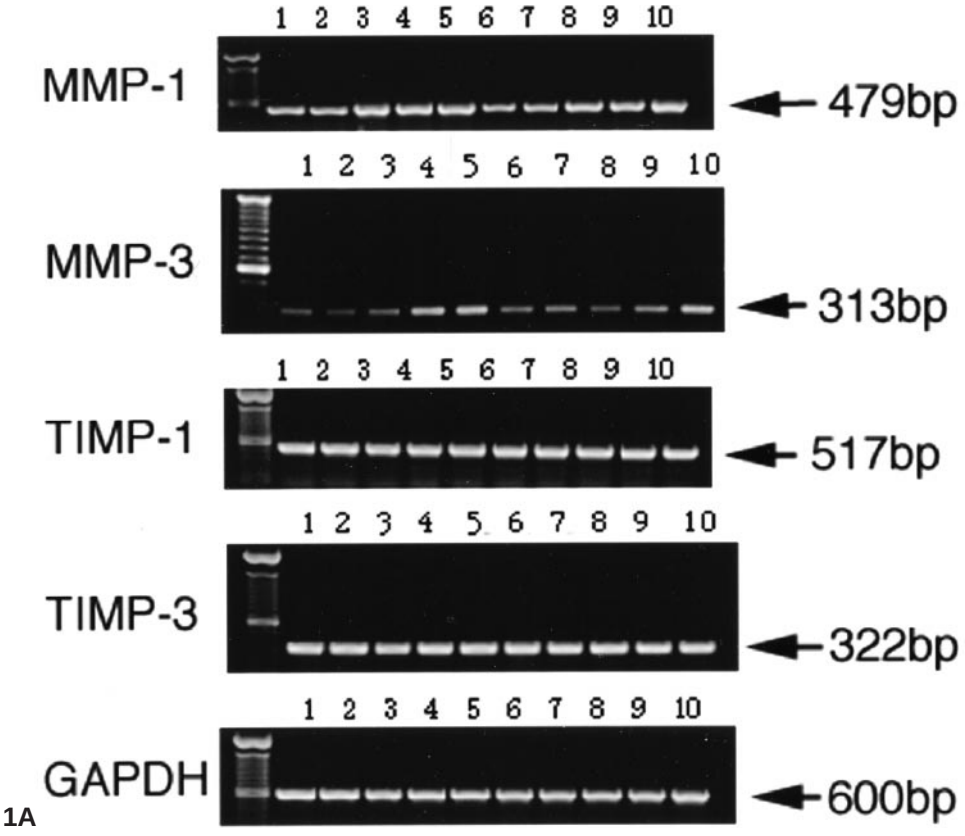
The following primer pairs specific for MMP-1, MMP-3 TIMP-1, TIMP-3 and GAPDH were used (5′-3′):

- GAPDH sense, CCACCCATGGCAAATTCCATGGCA
- GAPDH antisense, TCTAGACGGCAGGTCAGGTCCACC
- MMP-1 sense, GTATGCACAGCTTTCCTCCACTGC
- MMP-1 antisense, GATGTCTGCTTGACCCTCAGAGACC
- MMP-3 sense, TGCTTTGTCCTTTGATGCTG
- MMP-3 antisense, TTCCTTATCCGAAATGGCTG
- TIMP-1 sense, TTCCGACCTCGTCATCAGGG
- TIMP-1 antisense, ATTCAGGCTATCTGGGACCGC
- TIMP-3 sense, CTGACAGGTCGCGTCTATGA
- TIMP-3 antisense GGTCTGTGGCATTGATGATG

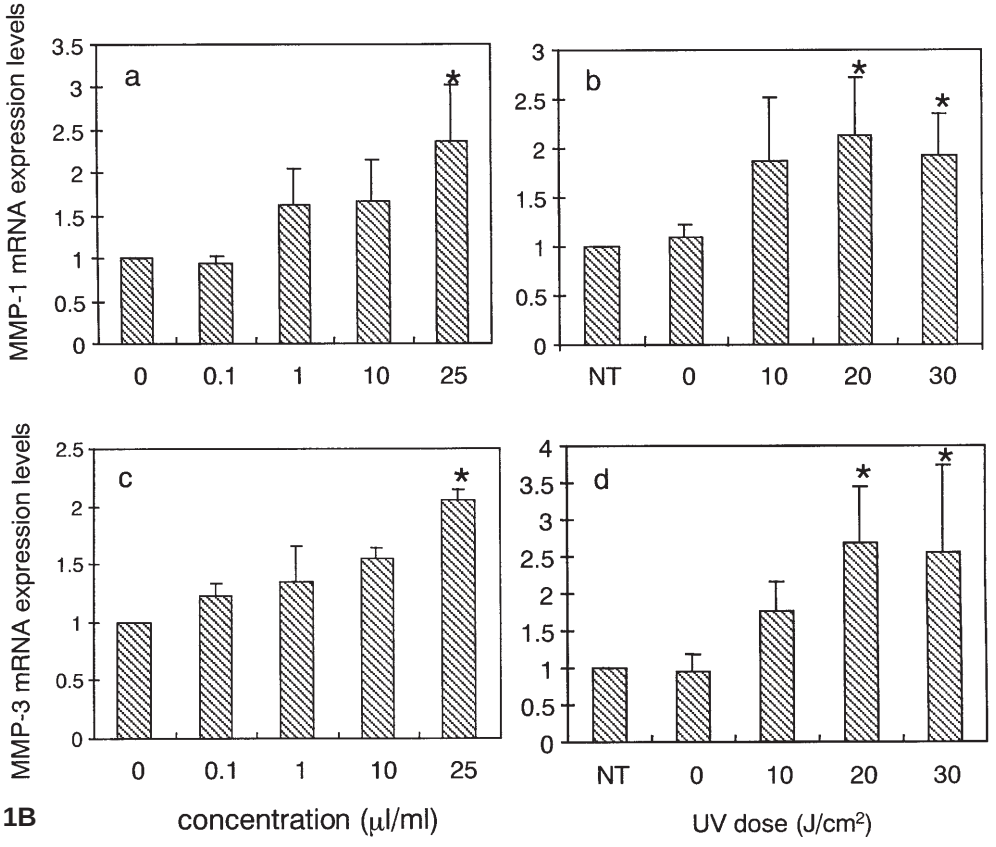
Parallel to each PCR, a PCR for GAPDH was performed as described above. Furthermore, PCR of each sample for each enzyme was carried out at least three times. Original gels were scanned for quantitative analysis by NIH image software which was downloaded from http://www.zippy.nimh.gov/pub/image. To ensure the identity of products, each cDNA was digested with the respective restriction endonuclease, and fragments were visualized on agarose gel by ethidium bromide staining. Lengths of the restriction fragments were compared with those deduced from published mRNA sequences of the respective MMPs and TIMPs.

Western blotting

The medium was harvested in equal volumes of 2 × SDS gel loading buffer (0.5 *M* Tris chloride, pH 6.8, 10% SDS, 20% glycerol, 0.2% bromophenol blue). The total protein content of the conditioned medium was measured using a micro-BCA protein assay (Pierce, Rockford, Ill.). Protein (20 μg per well) was resolved on 5–20% polyacrylamide ready gels (Bio-Rad, Hercules, Calif.) under nonreducing conditions. Proteins were electrophoretically transferred to a polyvinylidine difluoride (PVDF) membrane (Nihon Millipore, Tokyo, Japan). After the membranes were blocked in the nonspecific binding buffer (5% skimmed milk, 0.05% Tween 20 in Tris-buffered saline, pH 7.5) overnight, they were incubated with the designated primary antibodies for 2 h at room temperature. The following primary antibodies were employed: a monoclonal antibody to human type I collagen and a monoclonal



1A



1B

Fig. 1A, B **A** Expression of MMP and their TIMP mRNA induced by treatment with tobacco smoke extract and UVA1. The mRNA was prepared from fibroblasts following irradiation with UVA1 (*no. 1* no treatment, *no. 2* sham irradiation, *no. 3* 10 J/cm^2, *no. 4* 20 J/cm^2, *no. 5* 30 J/cm^2) or exposure to tobacco smoke extract (*no. 6* control, *no. 7* 0.1 μl/ml, *no. 8* 1.0 μl/ml, *no. 9* 10 μl/ml, *no. 10* 25 μl/ml). Reverse transcription and amplification of the generated cDNA by PCR were performed as described in Materials and methods. Amplified products of MMP-1, MMP-3, TIMP-1, TIMP-3 and the control gene GAPDH were visualized by ethidium bromide staining in agarose gel. The data are from one of three identical experiments. **B** Densitometric evaluation of the MMP-1, MMP-3, TIMP-1, and TIMP-3 generated from fibroblasts following stimulation with tobacco smoke extract (*a*, *c*), or UVA1 (*b*, *d*). The results (mean of three separate experiments) are expressed as the ratios of the signal of each PCR products to the corresponding GAPDH PCR product normalized to the no-treatment control samples. Data are means ± SEM. The significance of differences was determined by the Tukey-Kramer test and is indicated on each bar

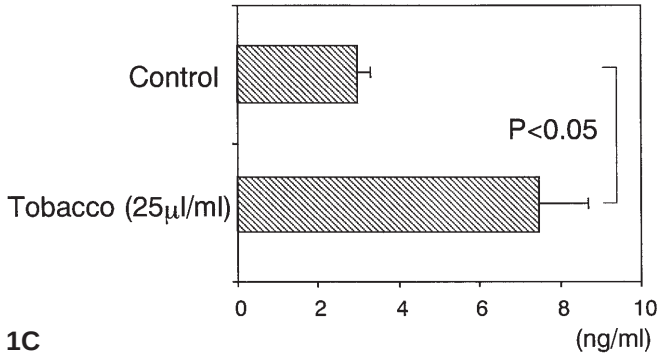


Fig. 1C MMP-1 protein was elevated following treatment with tobacco smoke extract as determined by ELISA. The level of MMP-1 following treatment with 25 μl/ml tobacco smoke extract was significantly higher than that of the control ($P < 0.05$). The results are presented as the means ± SEM

antibody to human type III collagen (both Fuji Chemical Industries, Toyama, Japan). The primary antibodies were reacted with antimouse peroxidase-conjugated IgG (DAKO, Denmark) for 1 h at room temperature and detected by chemiluminescence (ECL system; Amersham Pharmacia Biotech). Washed blots were exposed to hyper-film (Amersham Life Science, Little Chalfont, UK).

Detection of MMP-1 by enzyme-linked immunosorbent assay (ELISA)

After treatment with tobacco smoke extract, the medium was harvested and MMP-1 protein was determined by ELISA (Amersham Pharmacia Biotech, Little Chalfont, UK). The values were determined in duplicate.

Quantification of newly synthesized collagen

Fibroblasts were seeded in 96-well microculture plates at a concentration of 5000 cells in 100 μl cDMEM supplemented with 50 μg/ml L-ascorbic-acid (Katayama Chemical, Osaka, Japan) and 50 μg/ml beta-aminopropionitrile (Katayama Chemical). Prior to exposure to ^{3}H-proline in each well, fibroblasts were cultured in starvation medium (no FCS) overnight after reaching subconfluence. Cells were labeled with 18.5 kBq/ml ^{3}H-proline for an additional 24 h in cDMEM with 50 μg/ml L-ascorbic-acid and 50 μg/ml beta-aminopropionitrile. The ^{3}H-proline incorporation into pepsin-resistant, salt-precipitated extracellular collagen was then determined by the method of Webster and Harvey (Webster and Harvey 1979). Briefly, cell-associated ^{3}H-proline-labeled collagen was extracted by the addition of acetic-acid-pepsin following gentle shaking at 4 °C overnight and purified by salt precipitation at acid and neutral pH. The final precipitate was solubilized in 0.5 *M* acetic acid, incorporated into a scintillation cocktail and counted in a liquid scintillation counter (LS5801; Beckman, Fullerton, Calif.). The results from eight identically treated wells were expressed as disintegrations per minute. Cell numbers were determined by hemocytometer counting of trypsin-released cells present in a parallel assay without radioactivity.

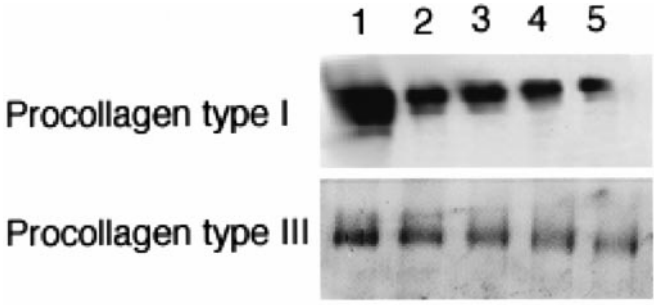


Fig. 2 Type I and type III procollagen degradation of human fibroblasts exposed to tobacco smoke extract. Supernatants from fibroblast cultures were harvested 24 h after treatment with tobacco smoke extract and subjected to Western blot analysis (*no. 1* control, *no. 2* 0.1 μl/ml, *no. 3* 1.0 μl/ml, *no. 4* 10 μl/ml, *no. 5* 25 μl/ml of tobacco smoke extract)

Results

Tobacco smoke extract induces MMP-1 and MMP-3, but not TIMP-1 and TIMP-3 expression, in human skin fibroblasts

The results of the RT-PCR analysis of the expression of MMP-1, MMP-3, TIMP-1, and TIMP-3 mRNA in the skin fibroblasts stimulated with tobacco smoke extract or UVA1 radiation are shown in Fig. 1 A, B. The fibroblasts treated with tobacco smoke extract or UVA1 radiation expressed higher MMP-1 and MMP-3 mRNA levels than control fibroblasts. MMP-1 mRNA expression was dose-dependently elevated by tobacco smoke extract (2.4-fold) at 25 μl/ml and by UVA1 (2.1-fold) at 30 J/cm^2 compared with the corresponding control. The expression of MMP-3 mRNA was also dose-dependently increased by tobacco smoke extract (2.1-fold) at 25 μl/ml and UVA1 (2.5-fold) at 30 J/cm^2. These differences were statistically significant ($P < 0.05$) at 25 μl/ml tobacco smoke extract or UVA1 at 20 and 30 J/cm^2 as compared with controls. In contrast, the expression of TIMP-1 and TIMP-3 mRNA remained unaltered by both stimulations.

Tobacco smoke extract induces MMP-1 protein in human skin fibroblasts

The protein levels of MMP-1 secreted into the supernatant of the tobacco treatment culture system, as measured by ELISA, were significantly higher following treatment with 25 μl/ml tobacco smoke extract compared with control ($P < 0.05$; Fig. 1 C).

Tobacco smoke extract inhibits the production of type I and type III procollagens

Production of collagens type I and type III was assessed by Western blotting in skin fibroblast medium treated with tobacco smoke extract. The protein recognized by

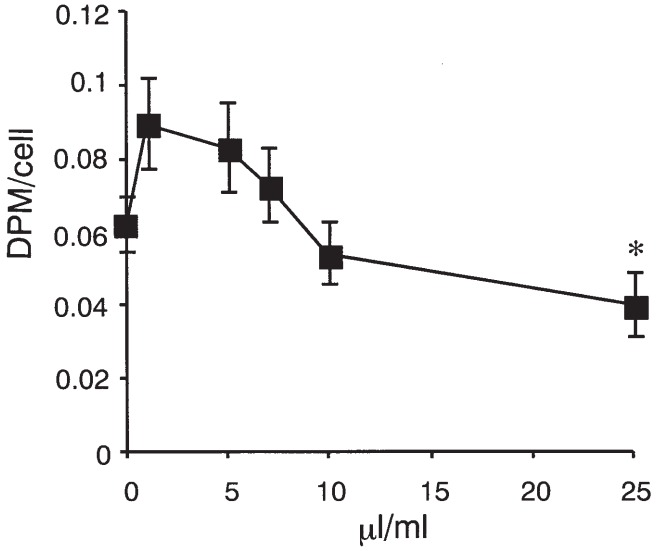


Fig. 3 Effects of tobacco smoke extract at various concentrations (0, 0.1, 10, 25 µl/ml) on collagen biosynthesis by human skin fibroblast cultures. Collagen biosynthesis by fibroblast microcultures during the final 24 h of treatment with tobacco smoke extract was assessed by ^{3}H-proline incorporation into pepsin-resistant salt-precipitated collagenous protein as described in Materials and methods. Data were confirmed in two additional experiments. The results are expressed as disintegrations per minute (± SEM) per cell

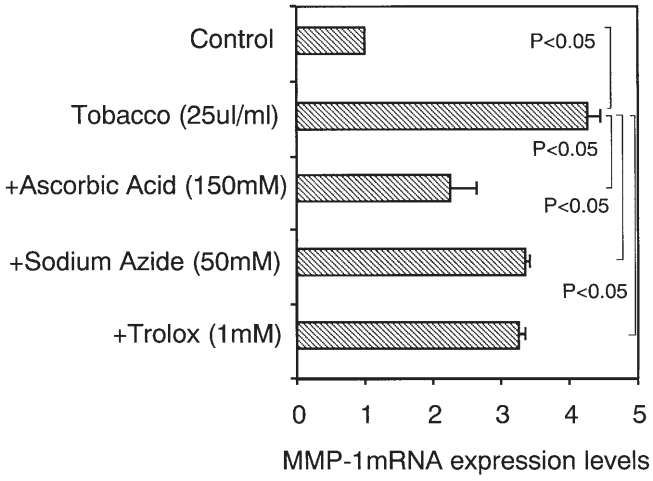


Fig. 4 Effects of different antioxidants on MMP-1 mRNA expression levels in human skin fibroblasts exposed to tobacco smoke extract. Human skin fibroblasts were exposed to 25 µl/ml tobacco smoke extract for 24 h and incubated at the same time with the following agents: 50 m*M* sodium azide, 150 µ*M* L-ascorbic acid or 1 m*M* Trolox. Gels were analyzed by semiquantitative NIH image software. The results are expressed as the means ± SEM

antibodies to type I and type III collagens migrated with a relative molecular mass of approximately 160–170 kDa, indicating that the fibroblast medium supernatant contained predominantly full-length procollagen. As shown in Fig. 2, treatment with different concentrations of tobacco smoke extract decreased the levels of both type I and type III procollagens present in the skin fibroblast supernatants. The maximal decreases in type I and type III procollagens induced by tobacco smoke extract, as assessed by densitometry, were 91.8% and 89.3%, respectively.

Collagen biosynthesis is decreased by tobacco smoke extract

The biosynthesis of new collagen was assessed in skin fibroblasts treated with tobacco smoke extract by measuring ^{3}H-proline incorporation into collagen. ^{3}H-proline incorporation was decreased in a concentration-related fashion at tobacco extract concentrations of 10 and 25 µl/ml (Fig. 3). The maximal decrease of 40.1% ($P < 0.05$) was seen with an extract concentration of 25 µl/ml.

Tobacco smoke extract-induced MMP-1 expression is inhibited by antioxidants

Reagents capable of quenching singlet oxygen were assessed for their capacity to modulate tobacco smoke extract-induced MMP-1 mRNA expression. MMP-1 mRNA expression in fibroblasts exposed to tobacco smoke extract was significantly decreased in the presence of 50 m*M* sodium azide (Katayama Chemical, Osaka, Japan), 150 µ*M* L-ascorbic acid, and 1 m*M* Trolox (Aldrich Chem Co, Milwaukee, Wis.) compared with the expression following tobacco smoke extract treatment alone ($P < 0.05$; Fig. 4).

Discussion

Normal human dermis consists primarily of an ECM of connective tissue collagen, which accounts for about 80% of the dry weight of the skin. Alterations in the amount of collagen or disturbance of its degradative pathway results in clinical symptoms of cutaneous aging.

This study demonstrated that the expression of MMP-1 and MMP-3 mRNA, ECM-associated members of the MMP gene family, was induced in cutaneous human skin fibroblast upon stimulation with tobacco smoke extract. Exposure to tobacco smoke has previously been found to increased MMP-1 mRNA levels in rat lungs in vivo (Morimoto et al. 1997). Similarly, nicotine, the active ingredient of tobacco, has been shown by Northern hybridization analysis to decrease type I procollagen mRNA in cardiac fibroblasts (Tomek et al. 1994). The expression of TIMP-1 and TIMP-3, however, remained unchanged in the present study. By inducing the expression of MMP-1 and MMP-3, but not the expression of TIMPs, tobacco smoke extract could alter the ratio in favor of the induction of MMPs and thereby cause a more degradative environment that produces loss of cutaneous collagen. Taken together, these results indicate that MMPs are primary mediators of connective tissue damage in skin exposed to tobacco smoke extract and thereby cause premature aging of skin in smokers. The functional relevance of the altered

balance between MMPs and TIMPs was demonstrated by protein analysis which showed that the content of type I and type III procollagens in supernatants from tobacco smoke extract-treated cells was significantly decreased.

We also found that the biosynthesis of new collagen was significantly decreased by tobacco smoke extract in cell layers. Tobacco has been shown to have a biphasic effect on collagen biosynthesis. At a low concentration (1 μl/ml), collagen synthesis by the treated fibroblasts was higher, whereas at a higher concentration (25 μl/ml) collagen synthesis was significantly decreased. Similarly, Chamson et al. have demonstrated that the rate of collagen synthesis as well as the types of collagen produced are affected (Chamson et al. 1982). Lenz et al. and Ramp et al. have examined the effects of tobacco on primary osteoblast-like cells, bone cells and sternal cartilage cells (Lenz et al. 1992; Ramp et al. 1991). They concluded that inhibition of collagen synthesis by tobacco is not specific to a certain cell type and that nearly all collagen-producing cells may be affected.

The studies presented here show that the reduced amounts of immunoreactive collagen (as much as nearly 90%) in Western blots was due to both decreased biosynthesis and a higher rate of degradation induced by the tobacco smoke extract. The elevation of MMP-1 protein levels was also confirmed in the medium of tobacco-treated cultures using an ELISA. Furthermore, tobacco smoke extract might affect several other enzymes and factors which could denature and undergo proteolysis including gelatinase (MMP-2, MMP-9) and collagenase-3 (MMP-13). These observations could provide evidence for suppressed collagen production and a higher rate of degradation leading to conditions favoring skin wrinkle formation.

Substantial progress has been made in recent years in advancing our knowledge about the biologic and molecular mechanism by which UVA1 radiation induces gene expression in human skin cells (Fisher and Voorhees 1998; Grether-Beck et al. 1997; Morita et al. 1997). UVA irradiation-induced gene expression is mediated through the generation of singlet oxygen via a pathway involving activation of transcription factor AP-2 (Grether-Beck et al. 1997). In the present study, sodium azide, L-ascorbic acid and Trolox (a water-soluble vitamin E derivative), which are potent quenchers of singlet oxygen and other reactive oxygen species, were found to significantly inhibit tobacco smoke extract-induced MMP-1 expression in fibroblasts. Among these antioxidants, L-ascorbic acid at a concentration of 150 μ*M* was clearly had the greatest effect. This is in line with the findings of Weber et al. indicating that vitamin C in human plasma is able to scavenge aqueous phase reactive oxygen intermediates resulting from the effects of tobacco smoke (Weber et al. 1996). The specificity of the quenchers employed in this study indicates that, as with UVA1 radiation-induced MMP expression, tobacco smoke extract-induced expression of these genes may be mediated at least in part through singlet oxygen generation. It should be noted, however, that the evidence for a role of singlet oxygen is circumstantial and does not exclude the possibility that other reactive oxygen species are involved as well.

Tobacco smoke contains at least 3800 constituents (Bartsch et al. 1993). Which constituent(s) contribute to the damage to connective tissue is still unclear. Further studies employing separation of these components by HPLC will be required to define the active ingredients in tobacco smoke extract responsible for premature aging of human skin. Alternatively, a specific antagonist, e.g. nicotinic receptor antagonist, could be another way to find the active component in tobacco smoke extract (Grando et al. 1996).

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