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Advances in Brief

Tumor Suppressor Maspin Is Up-Regulated during Keratinocyte Senescence, Exerting a Paracrine Antiangiogenic Activity

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Abstract

Cell senescence is a physiological program of terminal growth arrest, which is believed to play an important role in cancer prevention. Senescent cells secrete multiple growth-regulatory proteins, some of which can affect tumor growth, survival, invasion, or angiogenesis. Changes in expression of different senescence-associated genes were analyzed in cultured human skin keratinocytes (KCs) that underwent replicative senescence or confluence-induced accelerated senescence. Senescent KC cultures showed a strong increase in mRNA and protein expression of maspin, a member of serine protease inhibitor family and an epithelial cell tumor suppressor with anti-invasive and antiangiogenic activities. Immunohistochemical analysis of 14 normal human skin samples (age range from 3 months to 84 years) showed that maspin is expressed by KCs *in vivo* and that the extent and intensity of maspin expression in the skin is significantly ($P = 0.01$) correlated with chronological age. Antiangiogenic activity of maspin secreted by senescent KCs was investigated *in vitro* by testing the effect of conditioned media from different KC cultures on endothelial cell migration in the presence or absence of several angiogenic factors. Media conditioned by senescent cultures (undergoing replicative or accelerated senescence), but not by proliferating KCs, strongly inhibited the stimulation of endothelial cell migration by all of the tested angiogenic factors. Neutralizing antibody against maspin abrogated this effect of conditioned media. These findings indicate that senescent KCs exert a paracrine antiangiogenic activity, and maspin is the principal contributor to this potentially tumor-suppressive effect of cellular senescence.

Introduction

Cell senescence was originally described in normal human cells that cease proliferation in culture after a limited number of cell divisions, and it was proposed to reflect a process that occurs during organism aging (1). This phenomenon, known as replicative senescence, is mediated primarily by gradual changes of telomeres at the ends of chromosomes (2). Today, cell senescence is defined more broadly as a physiological program of terminal growth arrest, which can result not only from telomeric alterations but also from various forms of stress or inappropriate mitogenic signaling (*i.e.,* accelerated senescence; Ref. 2). Senescent cells do not divide even upon the addition of mitogens and acquire an altered phenotype, with large flat morphology and increased endogenous β-galactosidase activity (so-called senescence-associated β-galactosidase or SA-β-gal; Ref. 3). Terminal growth arrest of senescent cells is mediated by the up-regulation of cyclin-dependent kinase inhibitor proteins such as $p21^{Waf1/Cip1/Sdi1}$ and $p16^{Ink4A}$, as well as several other cell-cycle inhibitory genes (3, 4). The program of senescence, along with apoptosis, serves as an anticarcinogenic mechanism that stops the proliferation of normal cells that have experienced potentially carcinogenic damage (2), and the induction of accelerated senescence in tumor cells represents an important antiproliferative effect of anticancer drugs (4, 5).

In contrast to apoptosis, senescence does not destroy the cells but leaves them metabolically and synthetically active and therefore able to affect their environment. In particular, senescent fibroblasts were found to secrete proteins with known or putative tumor-promoting functions such as growth factors or proteolytic enzymes (3). Multiple genes encoding secreted factors with mitogenic, antiapoptotic, proteolytic, and angiogenic activities are coinduced in fibrosarcoma cells that overexpress $p21^{Waf1/Cip1/Sdi1}$ (6), as well as in colon carcinoma (7) and melanoma cells (8), which become senescent in response to chemotherapeutic drugs (4). Biological activity of senescence-associated tumor-promoting factors was demonstrated by the mitogenic and angiogenic activity *in vitro* of media conditioned by p21-overexpressing fibrosarcoma cells (6) and by the ability of normal senescent fibroblasts to stimulate the growth of transformed epithelial cells in coculture, *in vitro* and *in vivo* (9). This paracrine tumor-promoting activity of senescent fibroblasts suggests that cell senescence may not just prevent but also stimulate carcinogenesis.

On the other hand, colon carcinoma cells undergoing chemotherapy-induced senescence were found to up-regulate not only tumor-promoting factors but also several secreted factors with the opposite, tumor-suppressive activity (7). Such factors include insulin-like growth factor-binding proteins and maspin, a member of the serine protease inhibitor (serpin) family and a potent tumor suppressor with proadhesive, proapoptotic, anti-invasive, and antiangiogenic activities (10–12). Some of senescence-associated tumor-suppressive factors are also induced by DNA damage or by the overexpression of p53, leading to the bystander effect in coculture and conditioned media (CM) assays (13). The relative expression of tumor-promoting and tumor-suppressive genes in senescent cells varies depending on the cell and the inducer of senescence, and this balance is likely to determine whether senescence of normal and tumor cells has a predominantly pro- or anticarcinogenic effect (4).

Most of the data on the mechanisms and phenotypes of normal cell senescence has been generated in fibroblasts. During the past several years, we have been interested in defining the phenotype of senescent keratinocytes (KCs), identifying potential senescence bypass mechanisms involved in skin cancer and characterizing the molecular basis of angiogenic tissue responses in pathological skin disorders such as psoriasis (14–19). Learning more about the senescent phenotype of

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KCs *in vitro* may provide insight into the aging process for human skin, particularly because population aging is accompanied by increased number of skin cancers (20).

In the present study, we have asked which of the tumor-suppressive or tumor-promoting genes that are up-regulated in senescent tumor cells are associated with replicative senescence that develops after serial passage of normal human KCs or with accelerated senescence, which results from maintaining KC cultures at confluence (15). This analysis showed that the tumor-suppressor maspin is strongly up-regulated by senescent KCs *in vitro*. Moreover, maspin expression is also increased in aging skin *in vivo*. The induction of maspin in senescent KCs was additionally found to result in secreted antiangiogenic activity detectable by an *in vitro* assay. These findings suggest that normal senescent KCs are likely to have a paracrine tumor-suppressive activity and that maspin is a major contributor to this effect. Maspin expression by senescent KCs may potentially counteract the tumor-promoting paracrine effect of senescent fibroblasts and thereby minimize the age-dependent increase in the incidence of skin cancer.

Materials and Methods

Tissue Culture. Primary KCs were isolated from freshly excised neonatal foreskins as described (14). Briefly, normal KCs were grown using a low-calcium (0.07 mM) KC growth media (Clonetics Corp., San Diego, CA). KCs were harvested while they were either proliferating (<50% confluent), confluent, or senescent. Early confluency was defined by >60% of KC in the dish being juxtaposed or in contact with adjacent KCs. After reaching early confluency, parallel dishes were maintained up to 5 more days, with media changes every 2 days. At this time, >95% of KCs were in contact with adjacent cells giving the culture a crowded appearance. Senescent KC cultures were obtained after 60–70 population doublings and were unable to proliferate despite addition of fresh KC growth media, as described previously (15).

RNA Analysis. RNA was extracted from KCs in culture using Trizol reagent (Invitrogen, Carlsbad, CA) as described previously (19). After RNA isolation, semiquantitative reverse transcription-PCR was performed essentially as previously described (21), using β-actin as an internal normalization standard. Reverse transcription-PCR primer sequences and PCR conditions will be provided upon request.

Western Blot Analysis. To prepare whole cell protein extracts, cells were suspended in 1.5-ml Eppendorf vials with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid buffer and sonicated for 30 s followed by 30 min of vigorous shaking at 4°C as described previously (14). After centrifugation, supernatants were collected, and the protein concentration of each sample determined by Bio-Rad (Hercules, CA) protein assay. Immunoblotting assays were carried out by standard procedures using mouse monoclonal Abs against maspin (BD PharMingen, San Diego, CA) or β-actin (Sigma Chemical Co., St. Louis, MO). Bands were detected using horseradish peroxidase-labeled secondary Abs and enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ).

Tissue Samples and Immunohistochemistry. Normal human skin specimens were obtained from individuals undergoing elective plastic surgery procedures. Samples of normal appearing skin were fixed in formalin and embedded in paraffin following standard histological methods. Fourteen different tissue blocks were selected for study, including the following age groups: group 1, <15 years old (n = 4: 3 males, 1 female); group 2, 34–49 years old (n = 5: 2 males, 3 females); and group 3, 65–84 years old (n = 5: 3 males, 2 females).

Four-μm thick tissue sections were deparaffinized followed by antigen retrieval [10 mM citrate buffer (pH 6.0); microwaving 500 W; 15 min] as described previously (22). Primary Ab to detect maspin was obtained from Novocastra Laboratories Ltd. (clone EAW 24) and used at a final concentration of 10 μg/ml. Control Ab (pooled IgG) was obtained from BD PharMingen. After 1 h of incubation at room temperature, subsequent immunostaining was performed using avidin-biotin immunoperoxidase technique following the manufacturer's instruction (Vectastain, Vector Laboratories; Burlingame, CA). Slides were stained with the chromagen 3-amino-4-ethylcarbazole, generating a positive red reaction product and counterstained with hematoxylin.

After immunostaining, relative maspin levels were determined using a semiquantitative procedure in which the extent and intensity of staining was assessed as follows: extent: 0, no expression; 2+, one-third of KCs within epidermis positive; 4+, two-thirds of KCs within epidermis positive; and 6+, all epidermal KCs positive. Intensity: 0, no staining; 2+, faint staining; 4+, moderate staining; and 6+, strong staining. Thus, the range in the semiquantitative assessment scores was from 0 to 12.

Statistical analysis was performed using SPSS for Windows (SPSS 10.1; SPSS, Inc., Chicago, IL). The mean and SE for each age group was determined for the immunohistochemical staining scores and the Mann-Whitney U tests to compare the distribution of scores between groups. A two-sided P of $\leq$0.05 was considered statistically significant.

Detection of Maspin Secreted by KCs in CM. After 72 h, CM was collected from proliferating, confluent, senescent KC cultures and centrifuged to remove any residual cells and frozen at −80°C. Five-ml aliquots were thawed on ice and concentrated 10-fold using a Centricon-10 concentrator (Amicon, Beverly, MA). On the basis of protein concentration, equal quantities of protein were run on a 12% SDS-PAGE, proteins were transferred to Immobilon P membrane and probed with anti-maspin Ab (BD PharMingen). Relative levels of secreted maspin were determined by scanning laser densitometric analysis.

Endothelial Cell (EC) Migration Assay. The EC migration assay was performed as described previously (23). Briefly, human dermal microvascular ECs were starved overnight in media containing 0.1% BSA, harvested, resuspended into Dulbecco's Modified Eagle's Medium (DMEM) with 0.1% BSA, and plated on one side of a modified Boyden chamber (Neuroprobe). Test substances were added to the other side of the wells, and the cells were allowed to migrate for 4 h at 37°C. Membranes were recovered, fixed, stained, and the number of cells that had migrated from one side of the semiporous gelatinized membrane/10 high-power fields counted. Data are reported as the mean number of cells migrated/10 high-power fields (×400). Each substance was tested in quadruplicate. Growth factors (R&D Systems, Minneapolis, MN) were used where indicated at the following concentrations: vascular endothelial growth factor (100 pg/ml); interleukin 8 (40 ng/ml); and basic fibroblast growth factor (20 ng/ml). Neutralizing Abs for angiogenic cytokines (R&D Systems) were used at the following concentrations: antivascular endothelial growth factor (20 μg/ml); anti-interleukin 8 (20 mg/ml); and antibasic fibroblast growth factor (10 μg/ml). Neutralizing Abs for maspin (SC-8543; Santa Cruz Biotechnology) and a nonspecific IgG (BD PharMingen) were used at a concentration of 10 μg/ml.

Results

Induction of Maspin in Senescent KCs. We have asked which of the tumor-suppressive or tumor-promoting genes that were previously identified in different types of senescent tumor cells (6, 7, 24) are up-regulated in normal epithelial cells undergoing senescence. For this analysis, we have compared proliferating cultures of normal human KCs and KC cultures that became senescent after multiple passages or after being maintained at confluence for 3 days. As previously described (15), both types of nonproliferating KC cultures develop terminal growth arrest, express SA-β-gal and acquire other morphological and biochemical characteristics of senescent cells (data not shown). RNA from different KC cultures was used for reverse transcription-PCR assays for the expression of 27 senescence-associated genes, including secreted growth regulators, secreted tumor-promoting factors, intracellular growth inhibitors, and some others. The results of reverse transcription-PCR assays for the expression of some of these genes in two proliferating, one confluent, and one senescent KC culture are shown in Fig. 1*A*. Some of the tested genes showed increased expression in confluent and senescent KCs relative to proliferating cells, and such increases were especially common among secreted tumor-suppressive factors or intracellular growth inhibitors (Fig. 1*A*). These changes, however, were not always strong or clearly reproducible in different KC cultures. However, a strong and

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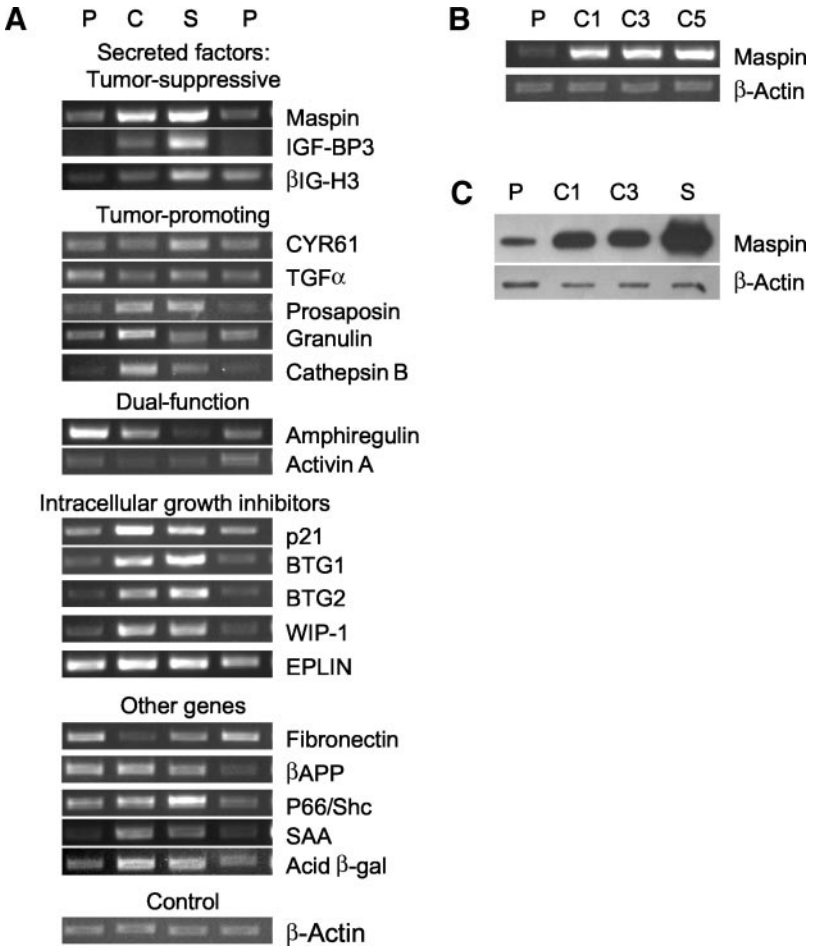

Fig. 1. Induction of maspin gene expression in senescent keratinocytes (KCs). *A*, reverse transcription-PCR analysis of RNA of the indicated genes in KC cultures that are proliferating (P; two independent cultures), confluent (C), or senescent (S). *B*, reverse transcription-PCR analysis of maspin RNA in KC cultures from the same individual that are proliferating (P), on days 1 (C1), 3 (C3), or 5 (C5) after reaching confluence. *C*, Western blot analysis of maspin protein in whole cell extracts of KC cultures derived from the same individual that are proliferating (P), senescent (S), or confluent on days 1 (C1), or 3 (C3) after reaching confluence.

reproducible increase in gene expression in both senescent and confluent cells was observed for tumor suppressor maspin, and we have therefore analyzed maspin expression in more detail.

Maspin RNA levels in confluent KC cultures increased over time at confluence (Fig. 1*B*), along with the development of the senescent phenotype (data not shown). In contrast to KCs, maspin RNA was nearly undetectable in proliferating or senescent fibroblasts or HT1080 fibrosarcoma cells (data not shown). To confirm the results of RNA analysis, whole cell extracts of KC cultures were examined by Western blotting using an anti-maspin Ab. Fig. 1*C* demonstrates low constitutive maspin levels in proliferating KCs and increased expression of maspin protein in confluent, as well as senescent KCs.

Maspin Expression in Human Skin Samples Is Age Associated. Maspin expression by KCs *in vivo* was characterized using formalin-fixed, paraffin-embedded sections of normal human skin obtained from 14 individuals of a wide range of ages. All samples were immunostained at the same time. The relative extent and intensity of maspin expression were assessed after immunostaining, using a semi-

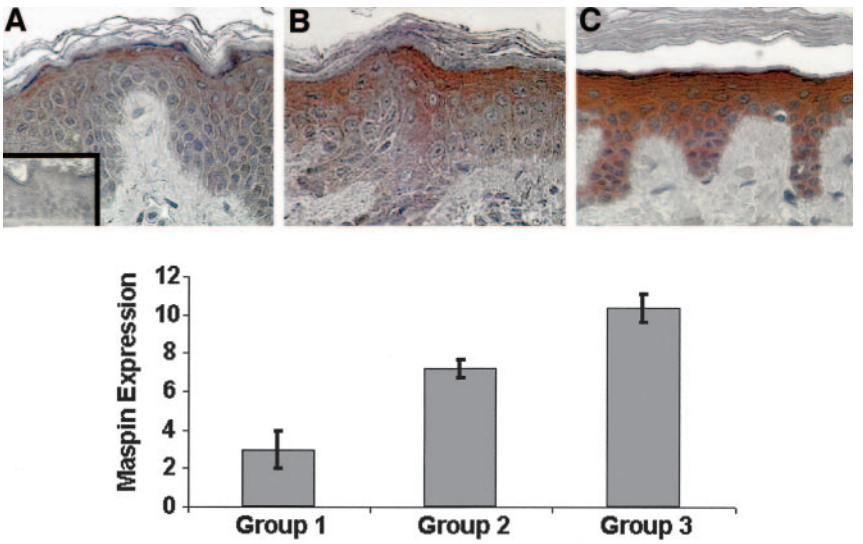

Fig. 2. Maspin expression in normal human skin. *Top panels* portray representative immunohistochemically stained sections to detect maspin. Immunoperoxidase stained, magnification, ×100. *A*, age group 1 with top one-third of epidermis with faint expression (score = 4); *insert*, IgG control stain. *B*, age group 2 with top one-third of epidermis with moderate expression (score = 6). *C*, age group 3 with entire epidermis with strong expression (score = 12). *Bottom panel* portrays semiquantitative assessment of maspin expression for all samples within each age group (mean ± SE). See "Materials and Methods" for details. Differences in maspin expression between group 1 and group 2 ($P = 0.01$). Differences in maspin expression between group 1 and group 3 ($P = 0.01$). Differences in maspin expression between group 2 and group 3 ($P = 0.02$).

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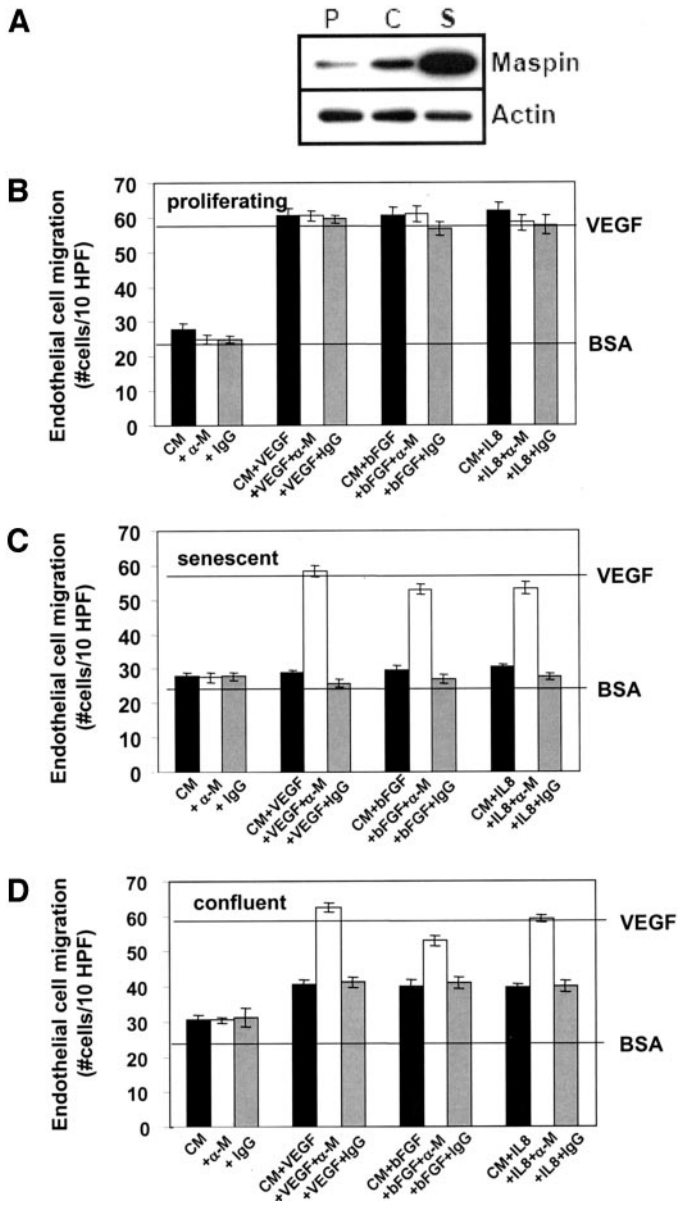

Fig. 3. Enhanced secretion of maspin by senescent keratinocytes (KCs) and effects of media conditioned by different KC cultures on *in vitro* angiogenesis. *A*, Western blot analysis of ×10 concentrated serum-free conditioned media (CM) to detect secreted maspin in cultures of proliferating (P) KCs, confluent (C) KCs, and senescent (S) KCs. See "Materials and Methods" for details. *B–D*, serum-free CM from (*B*) proliferating KC culture, (*C*) senescent KC culture, and (*D*) confluent KC culture were assayed for their ability to stimulate or inhibit EC migration alone or in the presence of anti-maspin antibody (α-M), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), or interleukin 8 (IL-8). See "Materials and Methods" for details. Values are reported as the number of cells migrated in 10 high-power field (HPF) + SE.

quantitative procedure on a scale of 1 to 12 (see "Materials and Methods"). Fig. 2, *top panels*, portrays representative maspin immunohistochemical staining results in normal human skin from young, middle-age, and older individuals. For data analysis, all of the donors were separated into three groups. In the youngest age group (0–15 years old, group 1), maspin expression was generally faint and confined to KCs in the upper epidermal layers. Individual maspin staining scores ranged from 0 to 4 in this group (mean = 3.0). By contrast in the group 2 samples (34–49 years old), maspin expression was of a stronger intensity and tended to include not only KCs in the upper epidermis but also mid-epidermal layer KCs with staining scores ranging from 6 to 8 (mean = 7.2). In the oldest set of skin samples obtained for group 3 (65–84 years old), maspin expression was strong and diffusely involved all epidermal layers of KCs with staining scores ranging from 8 to 12 (mean = 10.4). For all samples, there was no maspin staining of dermal fibroblasts, and IgG control staining revealed no significant positivity in either the epidermis or dermis (Fig. 2*A*, *inset*).

A summary of all of the scores for maspin expression in the three different age groups is portrayed in Fig. 2, *bottom panel*. A clear-cut trend is apparent in which increased maspin expression by epidermal KCs is observed when comparing the staining results for individuals in group 1, to group 2, and to group 3 (65–84 years old). These differences between groups were statistically significant (*P*s comparing group 1 to group 2 = 0.01; group 1 to group 3 = 0.01; and group 2 to group 3 = 0.02). Taken together, these immunohistochemical staining results indicate that there is a correlation in the extent and intensity of maspin expression by KCs in human skin samples with the chronological age of the individual.

Antiangiogenic Role for Maspin Secreted by Senescent KCs. To determine whether maspin was being secreted, CM was examined for maspin. Fig. 3*A* reveals that low levels of maspin could be detected in concentrated (×10) serum-free CM derived from proliferating KC cultures, but there was a 3- and 11-fold increase in maspin levels in the CM derived from confluent and senescent KC cultures, respectively. These levels of secreted maspin are consistent with the relative mRNA levels and whole cell extract-derived protein levels for proliferating *versus* confluent *versus* senescent KC cultures as depicted in Fig. 1. In the next set of experiments, the antiangiogenic role for the secreted maspin in CM was determined.

Antiangiogenic activity is the best documented tumor-suppressive function of maspin (25). To determine whether maspin produced by senescent KCs has biological activity, serum-free CM from proliferating, confluent, or senescent KC cultures were collected and added to human ECs in migration assays carried out in the presence or absence of different angiogenic factors. These EC migration assays were selected as they permit replicative analysis using relatively small amounts of CM and reflect angiogenic activity.

When tested alone, CM from any KC cultures failed to alter significantly the migration of human dermal microvascular endothelial cells *in vitro*, relative to the BSA control (Fig. 3*B–D*). The addition of either of the angiogenic factors vascular endothelial growth factor, basic fibroblast growth factor, or interleukin 8 to CM for proliferating KCs resulted in marked stimulation of endothelial cell migration (Fig. 3*B*). In contrast, the addition of vascular endothelial growth factor, basic fibroblast growth factor, or interleukin 8 to the CM from either senescent (Fig. 3*C*) or confluent KCs (Fig. 3*D*) results in only minor (Fig. 3*D*) or no stimulation of EC migration (Fig. 3*C*). These findings demonstrate that CM from the confluent or senescent cultures, but not from proliferating KCs, contains high levels of antiangiogenic activity.

To determine whether maspin, which is overexpressed by the senescent and confluent KCs, was responsible for blocking the ability of angiogenic factors to stimulate EC migration *in vitro*, a neutralizing Ab against maspin (10, 26) was added to CM from each KC culture. As expected, the addition of anti-maspin Ab to media conditioned by the proliferating KC did not alter EC migration in the presence or absence of angiogenic factors (Fig. 3*B*). The addition of anti-maspin Ab to CM from senescent or confluent KC in the absence of angiogenic factors also had no effect on EC migration (Fig. 3, *C* and *D*). However, the addition of anti-maspin Ab to CM from senescent or confluent KCs containing any of the angiogenic factors fully restored the ability of these factors to stimulate EC migration, blocking the antiangiogenic effect of CM (Fig. 3, *C* and *D*). These results demon-

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strate that maspin is the principal antiangiogenic factor secreted by senescent KC cultures.

Discussion

Senescence in normal human fibroblasts or fibrosarcoma cells is associated with increased expression of many secreted proteins that promote tumor growth and with paracrine tumor-promoting activities *in vitro* and *in vivo* (3, 6, 9). On the other hand, senescent epithelial tumor cells up-regulate not only tumor-promoting factors but also secreted tumor-suppressive proteins (7, 24). In the present study, we focused on maspin, a unique serine protease that can block tumor growth, invasion, and metastasis (11). The data presented in this report revealed that maspin is up-regulated in senescent cultures of normal human KCs *in vitro* and in aging KCs *in vivo* and that the induction of maspin secretion exerts paracrine antiangiogenic activity in CM of senescent KCs. These results suggest that secretion of maspin by senescent KCs may play an anticarcinogenic role in aging skin, not only by its well-known anti-invasive property but also by blocking angiogenesis.

Maspin has been characterized as a potent tumor suppressor, which is frequently down-regulated in breast and prostate cancers (10, 27, 28). Transfection of maspin into a transplantable breast cancer tumor line led to increased tumor encapsulation, less invasiveness, and a better prognosis (11), whereas maspin expression in a model of prostate cancer bone metastasis was shown to inhibit osteolysis, tumor growth, and angiogenesis (12). In addition to its other tumor-suppressive activities, maspin appears to have an apoptosis-sensitizing effect (10, 29). Many studies suggested that maspin, unlike most other serpins, does not possess a proteolytic inhibitory activity (30), but others have found that maspin inhibits urokinase-type plasminogen activator and that this activity correlates with the effect of maspin in inhibiting prostate cancer cell motility and invasion *in vitro* (11, 12). Maspin was also shown to function as a potent angiogenesis inhibitor (25) active in breast and prostate carcinomas (11, 29, 31–35). The antiangiogenic activity of this protein is maintained even after mutation of the reactive site loop of serpins, indicating that this function of maspin does not involve the inhibition of proteolysis (25). Maspin was recently shown to interact with type I and III collagen, and this effect on extracellular matrix was proposed to play a role in the antiangiogenic function of maspin (36).

On the cellular level, maspin was reported to be predominantly a soluble intracellular protein with some expression on the cell surface (37). Jiang *et al.* (32) demonstrated that maspin's effect on cell invasion and motility correlates with a location on the cell surface and in the pericellular space, whereas maspin's apoptosis-sensitizing effect correlates with intracellular localization. In the present study, we have found that *in vitro* antiangiogenic activity of maspin is transmitted through CM of maspin-producing senescent KCs, indicating that this activity is associated with the secreted maspin. Direct evidence for maspin secretion by KCs was provided by examining serum-free CM (Fig. 3*A*). Compared with relatively low levels detected in CM from proliferating KC cultures, there was a 3- and 11-fold increase in secreted maspin levels in CM-derived from confluent and senescent KC cultures, respectively. Although senescent cells secrete numerous factors into the media (4), the ability of anti-maspin Ab to abrogate the antiangiogenic activity of media conditioned by senescent KCs (Fig. 3*B–D*) indicates that this activity can be attributed entirely to maspin.

Maspin expression is not universal but appears to be restricted to specific cell types, including myoepithelial cells of breast, basal cells of prostate, mucosal and glandular epithelia of gastrointestinal tract, and stratified layers of skin. In some cases, its expression levels can even vary between locations within the same tissue type (37). In the present study, we have found that maspin RNA and protein expression in KCs correlate with senescence in cell culture and with chronological age in skin samples, providing an important new example of concordance between *in vitro* senescence and *in vivo* aging. The correlations that we have observed in skin samples, albeit statistically significant, are based on the analysis of only a small number of individuals (14 total), and therefore, they should be viewed as preliminary until a larger-scale study is performed. Our findings in human skin samples are in agreement with an earlier study that maspin is one of the proteins that show age-dependent expression in rat colon (38).

What is the mechanism of senescence- and age-dependent induction of maspin? Zou *et al.* (10) demonstrated that maspin gene transcription is strongly induced by DNA-damaging agents in different epithelial tumor cell lines. Maspin expression in aging tissues could therefore reflect epigenetic stabilization of stress effects accumulated over the lifetime, *e.g.,* through changes in promoter methylation. Importantly, maspin down-regulation in cancers has been associated with aberrant methylation of maspin promoter (28). Alternatively, high levels of maspin could reflect the pattern of transcription factors in senescent cells. Zou *et al.* (10) reported that maspin is directly induced by p53 (through the binding of p53 to its consensus binding site on the maspin promoter) and that p53 is required for the induction of maspin by DNA damage. We have found, however, that homozygous knockout of p53 in colon carcinoma cells only delays but does not abolish induction of maspin by a DNA-damaging agent (7). On the basis of the p53 immunostaining profiles (data not shown) and our previously published results on senescent KCs (19), it does not appear that maspin expression in senescent KCs requires functional p53. The maspin promoter, however, contains other transcription factor binding sites such as Ets, which was shown to regulate maspin expression in prostate (39) and AP-1, and we have previously shown that both Ets-1/2 and AP-1 regulate the expression of the senescence-associated cyclin-dependent kinase inhibitor p16 in KCs (15). Additional studies are indicated to define the molecular basis underlying the regulation of maspin expression by senescent KCs and aging human skin.

Senescence of normal human fibroblasts was shown to result in paracrine tumor-promoting activity *in vitro* and *in vivo* (9). Fibroblast senescence is associated with sequential up-regulation of cyclin-dependent kinase inhibitors $p21^{Waf1/Cip1/Sdi1}$ and $p16^{Ink4A}$. p21 expression in fibrosarcoma cells (6) and in normal fibroblasts (our unpublished data) activates transcription of several mitogenic, antiapoptotic, proteolytic, and angiogenic factors and produces mitogenic and apoptotic activities in CM. In the present study, we have observed that maspin gene expression was strongly induced in senescent KCs. Maspin is also induced in senescent colon carcinoma cells (7), but it is undetectable in senescent fibroblasts or fibrosarcoma cells (data not shown), suggesting that maspin is associated with senescence primarily in epithelial tissues. The induction of maspin by senescent KCs has resulted in paracrine antiangiogenic activity detectable by *in vitro* assays. It remains to be determined whether senescent KCs or other epithelial cells possess any other tumor-suppressive or tumor-promoting paracrine activities. Our findings, however, provide the first evidence that normal senescent cells possess an activity that can inhibit rather than promote tumorigenesis in the surrounding tissues. Such activities need to be taken into account in evaluating the role of cell senescence in carcinogenesis and cancer treatment.

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