BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **246,** 255–260 (1998)
ARTICLE NO. RC988593

Increased Hepatocyte Growth Factor Production by Aging Human Fibroblasts Mainly Due to Autocrine Stimulation by Interleukin-1

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Received April 3, 1998

Hepatocyte growth factor (HGF), also known as scatter factor is a pleiotropic factor that is mainly produced by mesenchymal cells and acts on cells of epithelial origin which express the HGF receptor c-Met. Here we demonstrate that production of HGF by human embryonic lung fibroblasts increased sharply after about 70% completion of their lifespan in culture, which is regulated at the transcriptional level. In addition, human skin fibroblasts from old donors, over 80 years, also produced more HGF than cells from young and middle-aged donors. The increased production of HGF by aging fibroblasts from human embryonic lung tissue is mainly due to autocrine stimulation by interleukin-1.

Hepatocyte growth factor (HGF), mainly produced by mesenchymal cells, has a heterodimeric structure consisting of a 60-kD heavy and a 30-kD light chain held together by a single disulfide bond, and exerts a variety of biological effects on cells of epithelial origin which express the HGF receptor c-Met, a 190-kD transmembrane protein possessing an intracellular tyrosine kinase domain. Although HGF was originally identified as a potent mitogen for hepatocytes, it enhances the proliferation of various types of cells and inhibits the proliferation of several tumor cell lines. HGF also induces motility and morphogenesis in hepatocytes and other extrahepatic cell types. Moreover, HGF is a potent angiogenic factor *in vitro* and *in vivo* [for reviews, see 1, 2].

It is important to investigate the regulatory mechanisms of *HGF* gene expression and its protein production to understand its pleiotropic actions. HGF is actively produced by human embryonic lung fibroblasts such as MRC-5, IMR-90 and M426, *ras*- or *sis*-transformed clones of mouse NIH 3T3 cells, vascular smooth muscle cells, and human leukemia cell lines [for reviews, see 1, 3]. We previously reported that the levels of HGF mRNA expression and HGF production by IMR-90 and MRC-5 cells were 3-fold higher at a population doubling level (PDL) of 70 than at a PDL of 40 [4]. In the present study, we examined whether HGF production in human embryonic lung fibroblasts would gradually increase with aging, or would abruptly increase from a specific cell age in culture. It was also determined whether similar events would occur *in vivo*, using human skin fibroblasts from young, middle-aged and old donors. In addition, we investigated the regulatory mechanisms through which HGF production increased in fibroblasts with aging in culture. As a result, we report here that HGF production by human embryonic lung fibroblast strains sharply increased after about 70% completion of their *in vitro* lifespans, and that increased production of HGF was also observed in human skin fibroblasts from old donors (over 80 years). Furthermore, we demonstrate that the increased production of HGF by aging fibroblasts is mainly due to autocrine stimulation by interleukin-1 (IL-1).

MATERIALS AND METHODS

Materials. Recombinant human IL-1α and IL-1β (rhIL-1α and rhIL-1β), and anti-human IL-1α and IL-1β rabbit antisera were kindly provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). 8-Bromo-cAMP was purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and culture. Human embryonic lung fibroblast strains, IMR-90 (lifespan, 73 PDLs) and MRC-5 (lifespan, 70 PDLs), were

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0006-291X/98 $25.00

purchased from American Type Culture Collection (Rockville, MD); TIG-7 (lifespan, 78 PDLs) [5] and WI-38 (lifespan, 54 PDLs) were donated by Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). These cell strains were used at PDLs of 39.3-72.2, 41.2-70.1, 14.6-75.6 and 27.6-46.3, respectively. Human skin fibroblast strains TIG-103, 104, 105, 106, 107, 111, 114, 118 and 121 were also donated by Tokyo Metropolitan Institute of Gerontology. The donors and lifespans of these cell strains are as follows: TIG-121, 8-month-old male, 52 PDLs; TIG-118, 12-year-old female, 66 PDLs; TIG-111, 34-year-old female, 47 PDLs; TIG-114, 36-year-old male, 58 PDLs; TIG-103, 69-year-old female, 49 PDLs; TIG-104, 72-year-old male, 41 PDLs; TIG-105, 72-year-old female, 37 PDLs; TIG-106, 80-year-old female, 37 PDLs; TIG-107, 81-year-old female, 43 PDLs. These skin fibroblasts were used at about 30-40% completion of their lifespans.

Cells were suspended in McCoy's 5a medium (ICN Biomedicals, Aurora, OH) supplemented with 10% fetal bovine serum, seeded onto 24-well plates at a density of 5×10^4 cells per well (2 cm^2) per 0.5 ml medium, or onto 6-well plates at a density of 2×10^5 cells per well (9-cm^2) per 2 ml medium, and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. For RNA extraction, cells were plated onto 80-mm dishes (50 cm^2) at a density of 1.25×10^6 cells per dish per 10 ml medium and cultured in the same way. The culture medium was renewed 1 day after seeding and thereafter every 2 days, unless otherwise indicated.

HGF production. On day 7, the medium was replaced with fresh medium or that containing the test cytokines and compounds. The conditioned media were collected after incubation for 48-96 h and stored at −20°C for HGF ELISA. Then some of the cultures were trypsinized for cell counting or were solubilized with 0.33 N NaOH for cellular protein determination after being washed three times with phosphate-buffered saline (PBS). Others were washed three times with PBS, and then the cell layers were scraped into ice-cold PBS containing 1.5 M NaCl, 0.039% Triton X-100, and 0.25% bovine serum albumin. Then the scraped cells were sonicated and centrifuged at 20,000g for 20 min at 4°C. These supernatants were also stored at −20°C for HGF ELISA.

A sandwich human HGF ELISA (Diagnostic Division, Otsuka Pharmaceutical Co. Ltd.) was performed at room temperature to quantitate the HGF contents in the conditioned media and in the cell extracts, as described previously [6]. The standard curve for human HGF was linear within the range of 0.025 to 5.0 ng/ml. HGF production per cell was determined by dividing the total amount of HGF in both the conditioned medium and the cell extract by the mean of the cell number before and after incubation for 48 to 96 h. Similarly, HGF production per milligram of cellular protein was determined by dividing the total HGF amount by the mean of the total cellular protein content in culture before and after 48-h incubation, which was determined by the method of Lowry et al. [7].

Analysis of gene expression by Northern blot. On day 7, the medium was replaced with fresh medium alone or that containing the test cytokines, and the cells were incubated for 48 h, unless otherwise indicated. They were then washed twice with PBS, scraped into 4 M guanidine thiocyanate solution containing 0.5% sodium dodecyl sulfate, 0.1 M 2-mercaptoethanol, and 1 M sodium citrate (pH 7.0), and stored at −80°C until RNA isolation and Northern blot analysis. Isolation of total RNA from cell monolayers by lysis in guanidine thiocyanate was carried out essentially as previously reported [4]. Poly(A)$^+$ RNA was separated from total RNA using an Oligotex-dT30 kit (Japan Roche Co. Ltd., Tokyo, Japan) per manufacturer's guidelines. Total and poly(A)$^+$ RNA samples (5-10 μg) were size-fractionated by electrophoresis on a 1% agarose-formaldehyde gel and transferred to a Hybond-N+ nylon membrane (Amersham International Plc, Little Chalfont, England) by the capillary method. After cross-linking under a UV light, membranes were prehybridized and then hybridized overnight with specific cDNAs (as indicated in the figures) that had been labeled with [^{32}P]dCTP using an Amersham *redi*prime DNA labelling system. The membranes were subsequently washed under high stringency conditions and exposed to X-Omat film (Kodak, Rochester, NY).

cDNA probes. The plasmids containing cDNA probes used to study gene expression were obtained from the following sources: plasmid pHFβ A-1 (human β-actin) [8] from Dr. K. Nose (Showa University, Tokyo, Japan), plasmids pUC118-hIL-1α (human IL-1α) and pGEM-3-IL-1β (human IL-1β) [9] from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan), and plasmid P met H (human c-*met*) [10] from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The *Bam* HI-*Kpn* I fragment (2.2 kbp) of human HGF cDNA [11] was obtained from Dr. N. Kitamura (Tokyo Institute of Technology, Yokohama, Japan). Hybridizations were performed using the following cDNA inserts: 2.2 kbp *Bam* HI-*Kpn* I fragment of human HGF, 1.4 kbp *Eco* RI-*Sal* I fragment of human c-*met*, 0.45 kbp *Eco* RI-*Hinc* II fragment of human IL-1α, 0.7 kbp *Pst* I-*Pvu* II fragment of human IL-1β, and 2.0 kbp *Bam* HI fragment of human β-actin.

RESULTS

Using human embryonic lung fibroblast strains, TIG-7 and WI-38, we first investigated whether HGF production by these cells would gradually increase with aging in culture, or increase abruptly from a specific cell age. Here we measured total amounts of HGF secreted into the medium and that in the cell extracts of TIG-7 cells at PDLs 14.6, 26.1, 37.4, 47.1, 56.4, 64.8, and 75.6. The total amount of HGF per either 10^6 cells, or per milligram of cellular protein, increased sharply after 72% completion of the *in vitro* lifespan (Fig. 1A). Similarly, production of HGF by WI-38 cells also increased remarkably after 66% completion of the *in vitro* lifespan (data not shown). Then we examined the expression of the *HGF* gene in these strains by Northern blot analysis. The increase in HGF mRNA in TIG-7 cells occurred in parallel with the rise in HGF protein production (Fig. 1B). The transcript in WI-38 cells also showed the same tendency (data not shown). Thus the production of HGF by these cell strains sharply increased starting from the characteristic cell age of about 70% completion of their *in vitro* lifespans, and was accompanied by an increase in the expression of the *HGF* gene.

Then we determined whether or not similar events would occur in human fibroblasts *in vivo*, using human skin fibroblast strains derived from young, middle aged, and old donors. Nearly confluent cell cultures were treated with 1 mM 8-bromo-cAMP, a potent enhancer of *HGF* gene expression [12], for 96 h to stimulate HGF production, because the amount of HGF constitutively produced by these strains was too small to be assayed by ELISA. As shown in Fig. 2, the cell strains derived from the oldest donors (80-81 years old) produced the most HGF among the 9 cell strains tested. In other words, HGF production by human skin fibroblasts sharply increased between the ages of 70 and 80 years.

To elucidate the mechanism of HGF induction in aging fibroblasts, we investigated IL-1 mRNA expression in IMR-90 and MRC-5 cells by Northern blot anal-

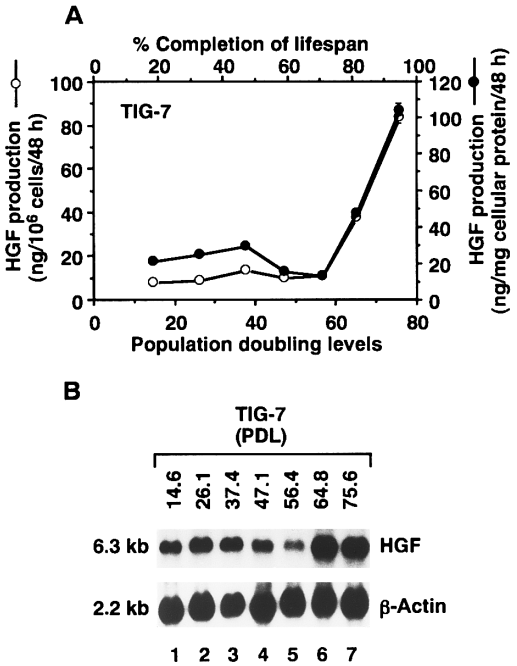

FIG. 1. HGF production by TIG-7 human embryonic lung fibroblasts sharply increases late in lifespan in culture. (A) Dynamics of HGF production by TIG-7 cells in the process of aging in culture. Cells at the indicated PDLs were cultured, and total amounts of HGF in the media and the cell extracts were measured by ELISA. Results are expressed as the mean from three wells. Vertical lines indicate SD. (B) Dynamics of expression of the *HGF* gene in TIG-7 cells with aging in culture. Cells at the indicated PDLs were cultured, and total RNA was isolated from the cultures and analyzed by Northern blot. The numbers on the left ordinate refer to the size (kb) of each specific signal. The intensity of β-actin mRNA was used as an internal control to compare the amount of loaded RNA between lanes.

ysis, because this cytokine has been demonstrated to up-regulate the expression of the *HGF* gene in MRC-5 cells [13] and to be produced by senescent diploid foreskin fibroblasts [14]. Old (PDL 67.8) IMR-90 cells expressed significant amounts of mRNA for both IL-1α and IL-1β, whereas young (PDL 41.8) cells expressed very little (Fig. 3A). We were also able to detect both mRNAs in old (PDL 68.0) MRC-5 cells but not in the young (PDL 48.1) cells (Fig. 3A). In addition, the old IMR-90 and MRC-5 cells expressed more IL-1β mRNA than IL-1α mRNA.

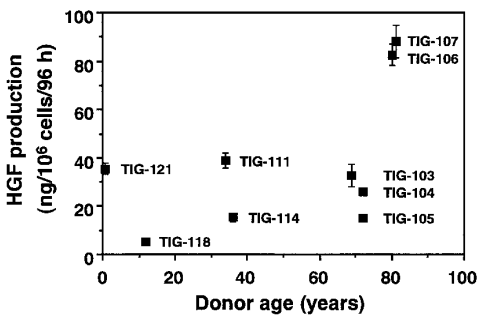

FIG. 2. Human skin fibroblasts from old donors produce more HGF than those from young and middle-aged donors. Human skin fibroblast strains at 30-40% completion of their lifespans were cultured, and the nearly confluent cultures were treated with 1 mM 8-bromo-cAMP for 96 h. Then, the total amounts of HGF in the media and the cell extracts were measured by ELISA. Results are expressed as the mean from three wells. Vertical lines indicate SD.

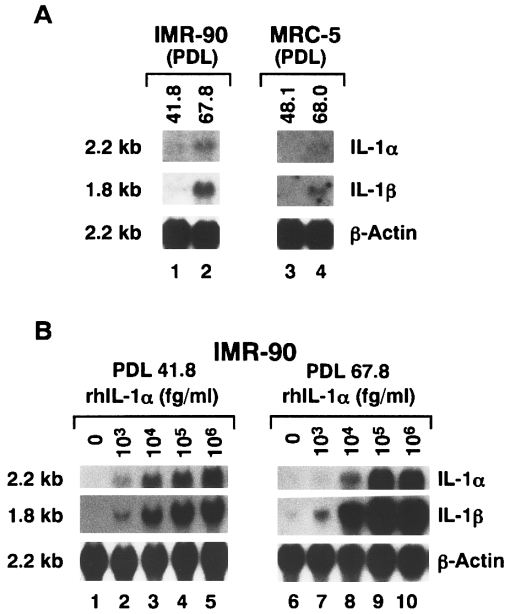

FIG. 3. Expression of mRNAs for IL-1α and IL-1β increases in IMR-90 and MRC-5 human embryonic lung fibroblasts with aging in culture. IMR-90 and MRC-5 cells at the indicated PDLs were cultured, and the nearly confluent cultures were treated for 48 h with or without rhIL-1α at concentrations of 10^3 to 10^6 fg/ml. Then, total RNA was isolated. (A) Poly(A)$^+$ RNA was separated from the total RNA samples extracted from the untreated cultures and analyzed by Northern blot. (B) Total RNA samples were used for Northern blot analysis. For other details see the legend of Fig. 1B.

It is known that *IL-1β* gene expression in human foreskin diploid fibroblasts is induced by IL-1α [14]. Then, we determined *IL-1* gene expression in young and old IMR-90 and MRC-5 cells treated with 10^3-10^6 fg/ml of rhIL-1α. Treatment with rhIL-1α dose-dependently increased the levels of both IL-1α and IL-1β mRNA in young and old IMR-90 cells (Fig. 3B) and in MRC-5 cells (data not shown). The IL-1α and IL-1β mRNA transcripts induced, however, were more abundant in the old cells than the young cells. These results show that IL-1α and IL-1β are constitutively expressed in aging fibroblasts.

Next we determined HGF induction by exogenous rhIL-1 in young and old human embryonic lung fibroblasts. Nearly confluent cultures of young (PDL 41.6) and old (PDL 72.2) IMR-90 cells were treated with 10^2-10^6 fg/ml rhIL-1α or rhIL-1β. As shown in Figs. 4A and 4B, both rhIL-1α and rhIL-1β dose-dependently increased HGF production by young IMR-90 cells. Their effects were detectable at 10^2 fg/ml and maximal

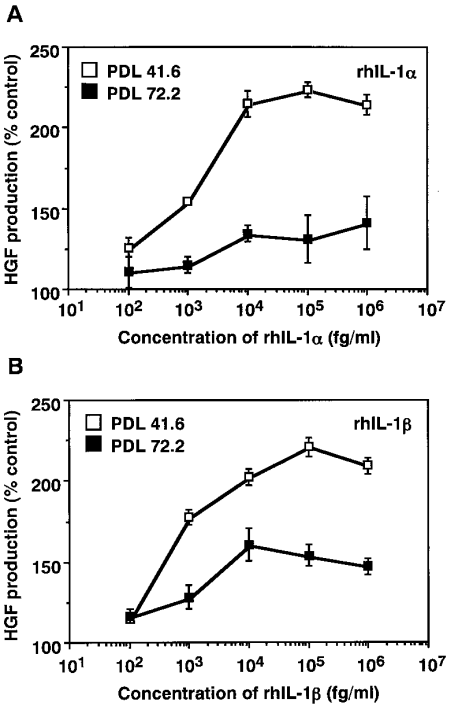

FIG. 4. Dose responses for rhIL-1α- and rhIL-1β-induced HGF production by young and old IMR-90 human embryonic lung fibroblasts. Nearly confluent cultures of young (PDL 41.6) and old (PDL 72.2) IMR-90 cells were treated for 48 h with or without rhIL-1α (A) or rhIL-1β (B) within a dose range of 10^2 to 10^6 fg/ml, and then the total amounts of HGF in the media and the cell extracts were measured by ELISA. Results are expressed as the mean of three wells. Vertical lines indicate SD.

at 10^5 fg/ml, showing about a 2.2-fold increase. HGF production by old IMR-90 cells was also enhanced dose-dependently by these cytokines, but their stimulation was maximal at 10^4 fg/ml and less potent as compared to the young cells (Figs. 4A and 4B). Furthermore, when tested with TIG-7 cells, induction of HGF by both IL-1s increased gradually up to PDL 47.0 and then decreased with age (data not shown).

Then we examined the effects of rabbit anti-human IL-1α and IL-1β antibodies on the production of HGF in IMR-90 and MRC-5 cells. The results are shown in Fig. 5. In this experiment, only the HGF levels in the cell extracts were determined because the HGF ELISA used was devised to detect rabbit anti-human HGF IgG with horse radish peroxidase-labeled goat anti-rabbit IgG antibody [6] and therefore we were unable to quantitate HGF in conditioned media containing rabbit IgG antibodies. However, the amount of HGF in the cell extracts of both IMR-90 and MRC-5 cell strains of about 40 to 70 PDLs was routinely about 50% of the total HGF produced as previously reported [4]. Treatment with anti-human IL-1α and anti-human IL-1β caused 24% and 54% decreases, respectively, in HGF production by old (PDL 70.2) IMR-90 cells (Fig. 5A). On the other hand, neither antibody affected HGF production in young (PDL 43.5) IMR-90 cells. Thus the amount of HGF produced in the old cells treated with anti-human IL-1β was almost the same as that produced in the untreated young cells. Figure 5A also shows the effects of IL-1 antibodies on IL-1β-induced HGF production. HGF, induced by exogenous rhIL-1β in both young and old cells as described above, was completely inhibited by anti-IL-1β to the level in the untreated young cells, and was partially inhibited by anti-IL-1α. Similarly, anti-IL-1β greatly reduced HGF production in old (PDL 67.9) MRC-5 cells, but not in young (PDL 47.6) cells (Fig. 5B). From these results, it is likely that the increased production of HGF by aging fibroblasts is mainly due to autocrine stimulation by IL-1.

Since HGF production remarkably increased in the human embryonic lung fibroblast strains, such as IMR-90, MRC-5, TIG-7, and WI-38, with aging in culture, we examined the expression in these strains of a proto-

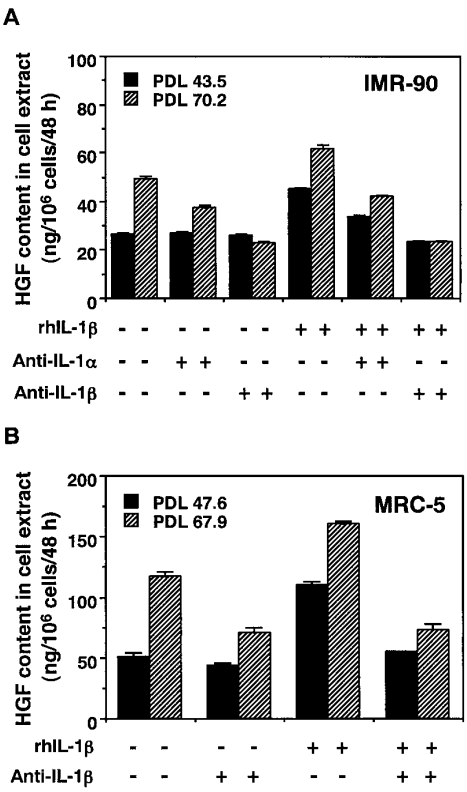

FIG. 5. Anti-human IL-1α and IL-1β antibodies inhibit HGF production by old IMR-90 and MRC-5 human embryonic lung fibroblasts but not that by young cells. IMR-90 (A) and MRC-5 cells (B) at the indicated PDLs were cultured, and the nearly confluent cultures were treated for 48 h with 1:500 dilutions of antisera against IL-1α or IL-1β, or normal rabbit serum in the presence or absence of rhIL-1β (10^4 fg/ml). Then the amount of HGF in the cell extracts was measured by ELISA. Results are expressed as the mean from three wells. Vertical lines indicate SD.

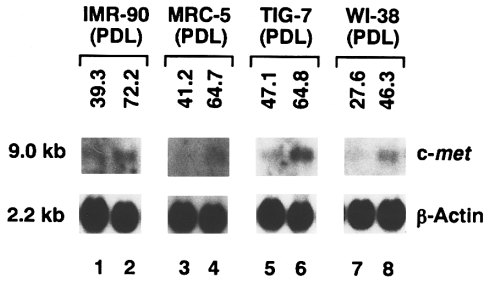

FIG. 6. Expression of the HGF receptor gene c-*met* increases in human embryonic lung fibroblasts with aging in culture. IMR-90, MRC-5, TIG-7 and WI-38 cells at the indicated PDLs were cultured, and total RNA was isolated from the nearly confluent cultures and analyzed by Northern blot. For other details see the legend of Fig. 1B.

oncogene c-*met*, which is identical to the HGF receptor gene. As shown in Fig. 6, the c-*met* expression also increased in these cells with aging in culture.

DISCUSSION

In the present study, we demonstrated that HGF production by human embryonic lung fibroblasts increased sharply after about 70% completion of their *in vitro* lifespans, which is regulated at the transcriptional level. Furthermore, the human skin fibroblast strains from old donors (over 80 years) produced more HGF than those from young and middle-aged donors. From these findings, it is likely that HGF production by human fibroblasts increases in the process of aging *in vivo* as well as *in vitro*.

The following lines of evidence support the notion that the increased production of HGF by aging fibroblasts is due mainly to autocrine stimulation by IL-1. (1) Expression of both IL-1α and IL-1β mRNA increased remarkably in old IMR-90 and MRC-5 cells, while that in young cells was quite low. (2) rhIL-1α and rhIL-1β added exogenously enhanced HGF production in young and old IMR-90 cells, induction in young cells being greater than that in old cells. (3) Anti-IL-1β suppressed HGF production in old IMR-90 cells to the level in untreated young cells. Anti-IL-1α was less effective, while neither anti-IL-1β nor anti-IL-1α affected HGF production in young cells, consistent with the negative mRNA expression of these cytokines. More effective inhibition by anti-IL-1β correlates with the higher expression of its mRNA than that of IL-1α mRNA.

A dramatic increase in the expression of mRNA for insulin-like growth factor-binding protein-3 (IGFBP-3) was observed in senescent human diploid fibroblasts compared with their early-passage counterparts [15]. IGFBP-3 binds insulin-like growth factor I (IGF-I) and inhibits the IGF-I-stimulated DNA synthesis of human diploid fibroblasts when co-incubated with IGF-I [16]. Thus, IGFBP-3, which is overexpressed in senescent human diploid fibroblasts, has the potential to inhibit DNA synthesis and cell growth. On the other hand, HGF is obviously a very powerful mitogen for hepatocytes and several other types of epithelial cells [for review, see 1] and is overexpressed in old IMR-90, MRC-5, TIG-7 and WI-38 cells as shown in the present study as well as our previous study [4]. In addition, expression of the HGF receptor gene c-*met* also increased in these cells with aging in culture. This may be due to HGF overexpression in these cells, since it has been reported to up-regulate c-*met* expression in primary rat fetal hepatocytes [17] and in A549 human lung adenocarcinoma cells [18]. Although there have been no reports that growth of the human embryonic lung fibroblasts is inhibited by HGF, it is of interest to investigate whether the increased HGF and its receptor c-Met would participate by autocrine mechanism in down-regulation of the growth of these cells with aging in culture.

IL-1 has an antiproliferative effect on normal cells, such as rat cardiac fibroblasts [19] and human endothelial cells [20]. Furthermore, senescent human endothelial cells have been shown to contain high amounts of the transcript for the IL-1α cytokine, a potent inhibitor of endothelial cell proliferation *in vitro* [21]. Treatment of human endothelial cell populations with an antisense oligodeoxynucleotide to the human IL-1α transcript prevents cell senescence and extends the proliferative lifespan of the cells *in vitro*. Conversely, removal of the IL-1α antisense oligomer results in generation of the senescent phenotype and loss of proliferative potential [21]. In the present study, old IMR-90 and MRC-5 human embryonic lung fibroblasts contained higher amounts of the mRNAs for IL-1α and IL-1β, as compared with their young counterparts. In addition, Osawa et al. [22] reported that exogenously added IL-1β induced p21/Waf1, a cyclin-dependent kinase inhibitor which regulates the cell cycle negatively [23], in WI-38 human embryonic lung fibroblasts and inhibited their growth. Thus, it is likely that IL-1, at least in part, participates by the autocrine mechanism in down-regulating the growth of human embryonic lung fibroblasts with aging in culture.

In conclusion, HGF production by human embryonic lung fibroblasts sharply increased after a characteristic cell age of about 70% completion of their *in vitro* lifespan, presumably due to autocrine stimulation by IL-1. In addition, an increase in HGF production was observed not only in fibroblasts aged *in vitro* but also in fibroblasts aged *in vivo*.

ACKNOWLEDGMENT

We thank Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan) for donation of rhIL-1α and rhIL-1β and anti-human IL-1α and IL-1β rabbit antisera.

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