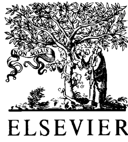
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Experimental Gerontology 36 (2001) 1317–1325

Experimental Gerontology

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Up-regulation of S100C in normal human fibroblasts in the process of aging in vitro

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Received 18 October 2000; received in revised form 19 February 2001; accepted 20 February 2001

Abstract

S100 proteins belonging to the EF-hand Ca^{2+}-binding protein family regulate a variety of cellular processes via interaction with different target proteins. Several diseases, including cancer and Alzheimer's disease, are related to a disorder of multifunctional S100 proteins, which are expressed in cell- and tissue-specific manners. We previously demonstrated that S100C could move to and accumulate in the nuclei of normal human fibroblasts but not in the nuclei of immortalized and neoplastic cells. In addition, we found that its nuclear accumulation resulted in suppression of DNA synthesis in normal cells at a confluent stage. In the present study, we investigated whether S100C was associated with cellular senescence in vitro. We found that S100C expression increased in normal human fibroblasts in the process of aging in culture and was accompanied by accumulation of its protein in the nuclei of senescent fibroblasts. In addition, the nuclear accumulation of S100C increased expression of a cyclin-dependent kinase inhibitor $p21^{Sdi1}$, a strong inhibitor of cell growth. These findings suggest that an increase in the cells having nuclear accumulation of S100C is closely related to the process of cellular senescence of normal human fibroblasts. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: S100C; Human senescent fibroblasts; Growth arrest

1. Introduction

S100C is an EF-hand type Ca^{2+}-binding protein and belongs to the S100 family. S100 protein was named after solubility of the first isolated protein in 100% saturated ammonium sulfate. It has been suggested that the S100 family, including S100C, plays important

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PII: S0531-5565(01)00097-3

roles in cell cycle regulation, differentiation, growth, and metabolic control (Allen et al., 1996; Marti et al., 1996; Scotto et al., 1998). By two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), we previously found that S100C was down-regulated in immortalized human cells (Sakaguchi et al., 2000, 2001). We found that S100C was phosphorylated and moved into the nuclei of normal cells when the cells reached confluence and stopped their growth. When intranuclear S100C functions in quiescent cells were blocked by anti-S100C antibody, DNA synthesis was resumed in the cells (Sakaguchi et al., 2000). To the contrary, when recombinant TAT-S100C fusion protein was compelled to enter the nuclei of immortalized KMST-6 cells, their DNA synthesis was remarkably inhibited (Sakaguchi et al., 2001). These findings indicated that nuclear S100C could control the cell growth arrest, especially contact inhibition of cell growth.

Normal human diploid fibroblasts loose division potential in the process of aging in culture, resulting in irreversible cessation of cell growth (Hayflick, 1965). This phenomenon is referred to cellular senescence. In general, cultured human fibroblasts in late passages exhibit specific characteristics, such as altered morphology with increased size, diminished mitogenic response to serum or growth factors, decreased migratory capacity, a higher incidence of chromosomal aberrations, expression of β-galactosidase, and increased levels of the cyclin-dependent kinase inhibitor $p21^{Sdi1}$ (for reviews: see Cristofalo and Pignolo, 1995; Young and Smith, 2000). Although the shortening of telomere has been proposed as the most probable mechanism of the replicative senescence of cells (Harley et al., 1990), the mechanisms of appearance of these senescent phenotypes are not completely elucidated yet. In the present study, we investigated to learn whether S100C associates with cellular senescence in vitro. We found that the expression of the S100C gene was up-regulated in normal human fibroblasts in the process of aging in culture and that S100C was observed in many nuclei of cells in senescent cultures. These findings indicated that nuclear S100C is possibly involved in the control of cell growth arrest and that an increase in the cells having nuclear S100C is associated with the senescent process of human cells.

2. Materials and methods

2.1. Cell cultures

Normal human fibroblast strains TIG-7 and WI-38 were cultured in a 5% CO_2 incubator at 37°C. Eagle's minimum essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Intergen, NY) was used. Characteristics of these cell strains have been described elsewhere (Yamamoto et al., 1991; Hayflick, 1965).

2.2. Two-dimensional gel electrophoresis

Protein was extracted from semiconfluent cultures. Cells were washed three times with PBS at 4°C, fixed with 10% trichloroacetic acid (TCA) for 40 min on ice, harvested by a rubber policeman, and centrifuged at 200 g for 5 min. The packed cells were lysed with a double volume of urea lysis buffer (8 M urea, 2.0% (v/v) triton X-100, 3.0% (w/v) CHAPS, 0.075% (w/v) DTT, 2.0% (v/v) ampholine, 1.0 mM PMSF, 1×10^{-3} mg/ml

aprotinin, 1×10^{-3} mg/ml leupeptin) and centrifuged at 12,000 g for 30 min. Protein concentrations were measured by Bradford's method (Bollag and Edelstein, 1991). Isoelectric focusing separation was performed as previously described using immobilized pH gradient (pH 5.5–6.5) gels (IPG) (Kondo et al., 1998). After separation of the proteins according to their isoelectric points with the IPG, the gels were directly placed onto 15% T SDS-polyacrylamide slab gels. The gels were run overnight at room temperature using a vertical electrophoresis system (Nihon Eido, Tokyo, Japan) for separation by molecular mass (MM). Proteins were detected on the gels by the glutaraldehyde-incorporating silver staining method (Kondo et al., 1998).

2.3. Immunoblot analysis

Immunoblotting was performed as previously reported (Sakaguchi et al., 2000), using a rabbit anti-human S100C antibody built up in our laboratory, goat anti-human $p21^{Sdi1}$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or goat anti-human actin antibody (Santa Cruz Biotechnology) followed by horseradish-peroxidase-conjugated anti-rabbit or goat IgG antibody (MBL, Nagoya, Japan). Then, S100C, $p21^{Sdi1}$, and actin were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotechnology, Tokyo, Japan).

2.4. Northern blot analysis

Total RNA was isolated by the acid guanidinum thiocyanate/phenol–chloroform method. Northern blot analysis was performed as previously described (Tsuji et al., 2000). Briefly, 20 μg RNA of each sample was fractionated in 1.0% agarose gels and then transferred to Nytran Plus nylon membranes (Amersham Pharmacia Biotechnology). Entire coding regions of S100C, and 28S rRNA were amplified by RT-PCR, and then the PCR products were used as probes.

2.5. Data analysis

At least two reproducible 2-D gels, Western blots, and Northern blots were obtained for each sample. 2-D gels and these blots were digitized with an EPSON scanner at 300 dots per inch. S100C spots and bands were detected and quantified with Bio-Rad Melanie II software on the basis of their relative volume, i.e. the S100C spot and band volumes divided by the reference volumes of actin spot or band. Thus, relative density of S100C was calculated as % volume of the internal control actin.

3. Results

3.1. S100C content in fibroblasts aged in vitro

Fig. 1 shows 15% T 2-D gel patterns of silver-stained proteins from normal young TIG-7 cells and their senescent counterparts at semiconfluence. The cellular senescence was judged by BrdU incorporation and β-galactosidase staining (data not shown). As shown in Fig. 1, S100C (pI 6.2 and MM 11 kDa) content was remarkably higher in the senescent

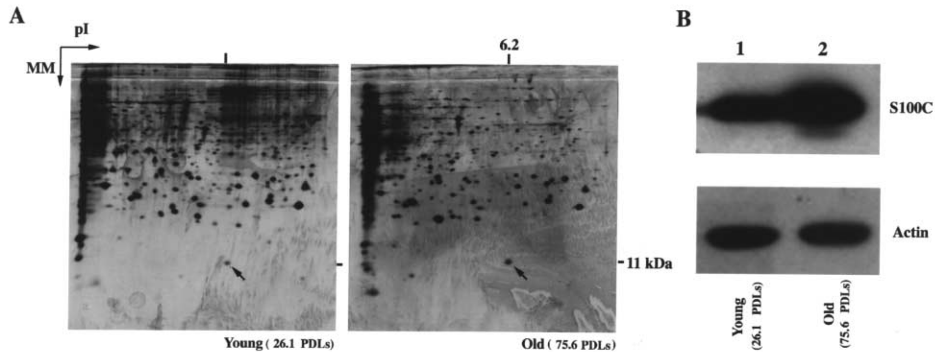

Fig. 1. A: 2-D gels of proteins from normal young TIG-7 cells at 26.1 population doubling level (PDL) (left) and their senescent counterparts at 75.6 PDL (right) in the semiconfluent culture. Arrows show S100C protein whose content was increased in the senescent cells compared with that in their young counterparts. The isoelectric point (pI) and molecular mass (MM) of the protein were 6.2 and 11 kDa, respectively. B: Immunoblot analysis of S100C in young and senescent TIG-7 cells at 26.1 and 75.6 PDLs, respectively. Actin content was also analyzed as an internal control.

TIG-7 fibroblasts than in the TIG-7 young fibroblasts (Fig. 1A, arrows). By direct sequencing, we confirmed that the protein was human S100C (Sakaguchi et al., 2000). Densitometric analyses of silver stained 2 D-gels (Fig. 1A) and Western blots (Fig. 1B) showed that there was five-fold increase in the S100C protein volume in the senescent TIG-7 cells as compared with the young cells.

3.2. Change in S100C expression in fibroblasts in the process of aging in culture

To investigate the dynamics of S100C expression in normal human fibroblasts in the process of aging in culture, we performed Northern blot analysis of S100C mRNA in TIG-7 and WI-38 cells at PDLs of 14.6–75.6 and PDLs of 24.3–39.4, respectively. S100C expression in TIG-7 cells increased in parallel with their population doubling levels (Fig. 2). A similar pattern of S100C up-regulation was also observed in WI-38 cells in the process of aging in culture (Fig. 2). The expression levels of S100C mRNA in senescent TIG-7 cells at 75.6 PDL and WI-38 cells at 39.4 PDL were 10-fold and nine-fold higher than those in their young cells at 14.6 and 24.3 PDLs, respectively.

We previously showed that S100C content in young fibroblasts remarkably increased at a high cell density, namely a cell-density-dependent quiescent stage. To evaluate cell-density-dependent changes of S100C content in the nearly senescent TIG-7 cells at 69.0 PDL and its young counterparts at 17.6 PDL, we compared the expression levels of S100C protein between young and old cells during cell growth after seeding at the same inoculum size. As shown in Fig. 3, doubling times of the young and old cells were 31.6 and 67.0 h, respectively. The saturation density of the young cells was four-fold higher than that of the old cells. Interestingly, the old cells already expressed a large amount of S100C protein at the low cell density as early as two days after seeding, whereas the expression level of S100C was low in the young cells at this early stage. The ratio of S100C band intensities in the young and old cells on day 2 after plating was 1:5. Thereafter, S100C in the old cells

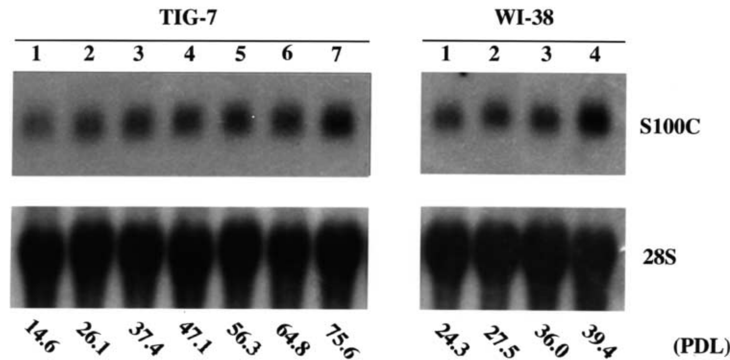

Fig. 2. Northern blot analysis of the steady-state expression levels of S100C mRNA in normal human TIG-7 and WI-38 fibroblasts. In vitro life spans of TIG-7 and WI-38 cells were 75.6 and 39.4 PDLs, respectively.

further increased. There was, however, no significant difference in S100C contents between the confluent old and young cells on day 10 after plating. These results indicate that S100C serves as a mediator of growth downregulation in cells. A cyclin-dependent kinase inhibitor $p21^{Sdi1}$, a strong growth inhibitor, also showed the same kinetic patterns as S100C in both the young and old cells (Fig. 3). Taken together, these findings show that there are more growth arrested cells in senescent cultures than in young cultures.

3.3. Subcellular distribution of S100C in senescent cells in vitro

We previously demonstrated that nuclear accumulation of S100C caused an inhibition of DNA synthesis in normal human KMS-6 fibroblasts, their immortalized KMST-6 cells, and neoplastic HeLa cells (Sakaguchi et al., 2000, 2001). These results indicate that there is a close relationship between nuclear S100C and cell growth arrest. Thus, we hypothesized that S100C would also accumulate in the nuclei of cells aged in vitro. To investigate the localization of S100C in senescent cells, we performed subfractionation analysis and immunostaining of S100C in TIG-7 cells. As a result, S100C was detected in the nuclear fraction of senescent TIG-7 cells (Fig. 4A, right). In addition, immunocytochemical analysis also showed that S100C was present in the nuclei of the senescent cells, even if they were not in the contact inhibition of cell growth (Fig. 4B, right). However, nuclear accumulation of S100C was not observed in their young counterparts (Fig. 4A, left and 4B, left).

4. Discussion

In order to clarify the relationship between nuclear accumulation of S100C and cell growth arrest, we previously investigated the functions of nuclear S100C in cells by following two strategies. First, when anti-S100C antibody was microinjected into the cytoplasm of confluent quiescent normal cells, anti-S100C antibody formed a complex with S100C protein and moved into the nuclei, and did the cells incorporate bromodeoxyuridine (BrdU) into their nuclei. Second, we artificially compelled the exogenous

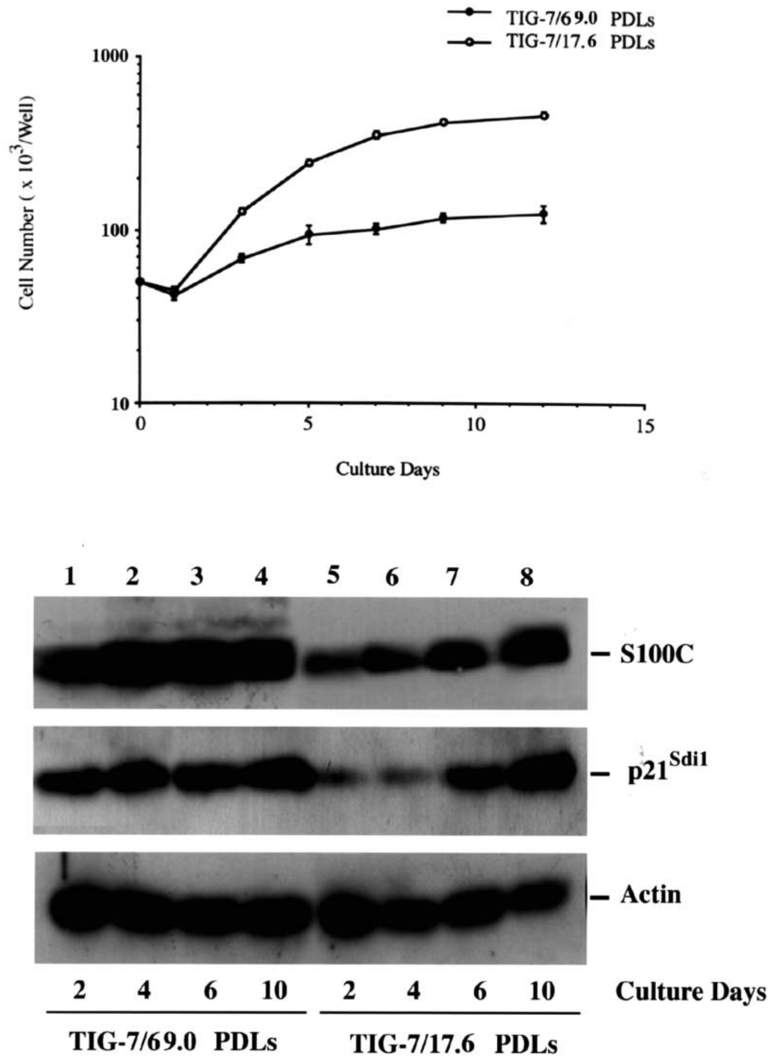

Fig. 3. Relationship between cell growth activity and S100C content in young and nearly senescent TIG-7 cells. Top: Growth curves of young and nearly senescent TIG-7 cells at 17.6 and 69.0 PDLs, respectively. Cells were plated at 5×10^4 cells per well in 24-well culture plate. Cells were detached by trypsinization and counted at the indicated times. Each value is the mean ± standard error from triplicate wells. Bottom: Immunoblot analysis of S100C in young and nearly senescent TIG-7 cells at 17.6 and 69.0 PDLs. $p21^{Sdi1}$ content was shown as a senescent and quiescent marker. Actin content was also analyzed as an internal control.

S100C to move into the nuclei of neoplastic HeLa cells, in which S100C was always localized in the cytoplasm, to determine whether such intranuclear S100C also could inhibit DNA synthesis of the cells. Namely, we transfected a tetracycline (Tet)-regulative *S100C-NLS* (nuclear localization signal) gene into the Tet-Off HeLa cells (Clontech Japan, Tokyo) and obtained a clone (Tet-Off HeLa/pTRE S100C-NLS) expressing S100C-NLS

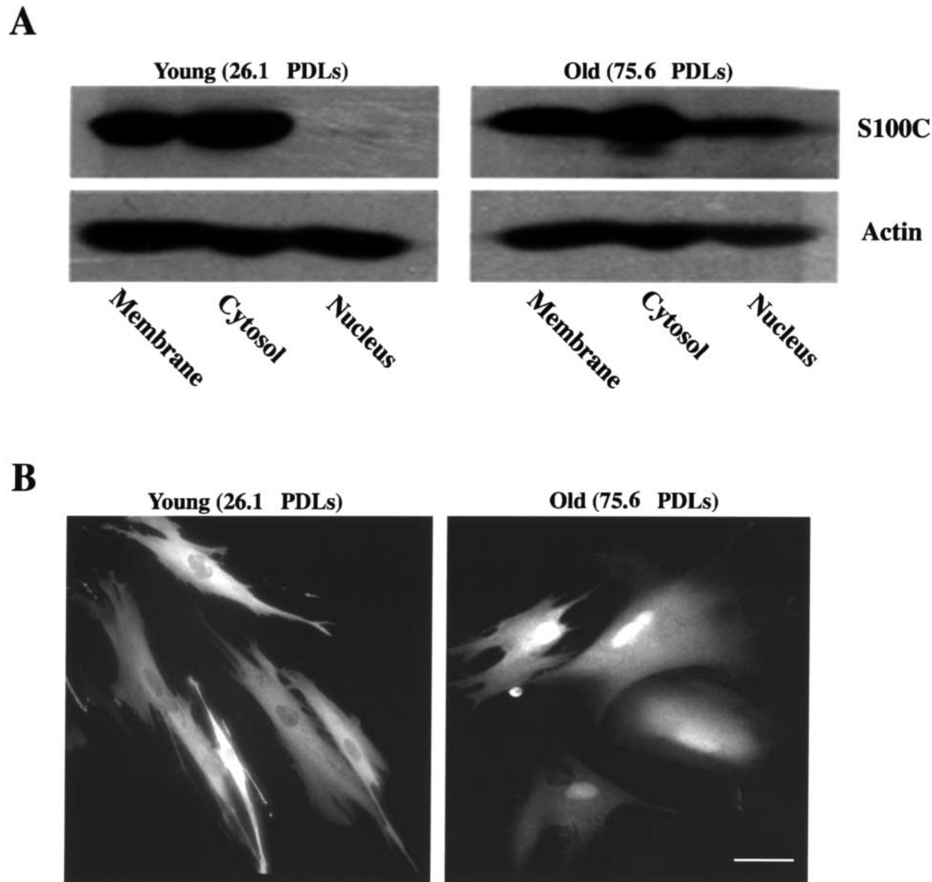

Fig. 4. A: Immunoblot analysis of S100C in membrane, cytosol, and nuclear fractions of young and senescent TIG-7 cells at 26.1 and 75.6 PDLs, respectively. S100C appeared in the nuclear fraction of the senescent cells but not in that of their young counterparts. B: Immunocytochemical staining of intracellular S100C. Left: Exponentially proliferating young TIG-7 cells at 26.1 PDL. Right: Senescent TIG-7 cells at 75.6 PDL. The intracellular S100C was stained with rabbit anti-human S100C IgG antibody and a fluorescence-labeled secondary antibody, TRITC-conjugated goat anti-rabbit IgG antibody. Scale bar represents 20 μm.

fusion protein. By removing tetracycline from the culture medium, the transfected cells stably expressed a significant amount of S100C-NLS fusion protein that was accumulated in the nuclei of the Tet-Off HeLa/pTRE S100C-NLS cells, resulting in inhibition of DNA synthesis of the cells (Sakaguchi et al., 2000). These two approaches demonstrated that nuclear S100C could act as a cell growth suppressor.

In the present study, we examined the dynamics of S100C expression in normal human fibroblasts in the process of aging in culture. Senescent fibroblasts expressed more S100C than did their young counterparts. In addition, S100C accumulated in the nuclei of the senescent cells to a greater extent than in the nuclei of their young counterparts. The nuclear accumulation of S100C was accompanied by an increase in expression of a strong

growth inhibitor $p21^{Sdi1}$ in senescent cells. However, it remains to be studied whether or not expression of $p21^{Sdi1}$ is induced by the nuclear accumulation of S100C protein.

Our present findings on S100C protein are consistent with some other S100 family proteins. For example, S100A2 is down-regulated in breast tumors and a variety of other neoplasms, suggesting that it could be a tumor suppressor (Lee et al., 1991; Pedrocchi et al., 1994; Wicki et al., 1997). Furthermore, S100B is also a growth inhibitor that is expressed specifically in neuronal cells in the brain, and this protein is closely associated with Alzheimer's disease (Marshak et al., 1991; Sheng et al., 1994). It is noteworthy that approximately 40% identity exists between S100C, S100B and S100A2 at the amino acid level and that most of the other amino acids are homologous, strongly suggesting that they have similar structures and functions.

In conclusion, it is likely that nuclear S100C is associated with the growth arrest of human fibroblasts at the senescent stage. To further definitively clarify the function of S100C in growth arrest and senescence, we are studying functions of S100C protein by regulating its expression in the cells.

Acknowledgements

We thank Dr K. Kaji for giving us normal human fibroblast strains (TIG-7 and WI-38).

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