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CELL CYCLE-DEPENDENT LOCALIZATION OF TISSUE INHIBITOR OF METALLOPROTEINASES-1 IMMUNOREACTIVITY IN CULTURED HUMAN GINGIVAL FIBROBLASTS

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ABSTRACT

Localization of the tissue inhibitor of metalloproteinases-1 (TIMP-1) was investigated in cultured human gingival fibroblasts by immunohistochemistry. TIMP-1 immunostaining was observed in the cytoplasm of a majority of cells, and in the nucleus of some cells. Depletion of fetal calf serum (FCS) from the culture medium reduced the density of immunoreactive TIMP-1 in the nucleus more remarkably than in the cytoplasm, and the following FCS-induced cell growth was accompanied by a recovery of immuno-reactive TIMP-1 in both the nucleus and the cytoplasm. The frequency of the nuclear TIMP-1-positive cells changed in line with that of PCNA-positive cells and was always much higher than that of S-phase cells, which were estimated by a pulse labelled 5-Bromo-2' deoxyuridine (BrdU). These results suggest a localization of TIMP-1 or a related substance in the nucleus of proliferating human fibroblasts, and its depletion from the nucleus due to an arrest of cell growth.

Key Words: TIMP-1, Human gingival fibroblast, Nuclear localization, Cell cycle

INTRODUCTION

The tissue inhibitor of metalloproteinases-1 (TIMP-1) is a 28 KDa secreted glycoprotein that binds and inactivates a number of matrix metalloproteinases, including interstitial collagenase, stromelysin and gelatinase, forming stable complexes with Kd values of around 10^{-10}. It is found in the connective tissue and in serum or tissue fluid.[1-7] Since metalloproteinases provoke the degradation of extracellular matrix, TIMP-1 also acts as a counterpart for enzymes in the regulation of turnover and remodeling of extracellular matrix. The depletion of a specific inhibitor or imbalance of protease/inhibitor activity may result in the tissue destruction, a common feature of tumour invasion and arthritis.

In 1985, Docherty et al.[8] reported on the cDNA of human TIMP-1, and found that it is identical to that of a protein which has been referred to as EPA (a cytokine with erythroid-potentiating activity). The actual erythroid-potentiating activity of TIMP-1 was then demonstrated on human bone marrow cells.[9] In addition, TIMP-1 has been shown to possess potent growth-promoting activity for a wide range of human and bovine cells.[10] TIMP-1 is produced by fibroblasts[5,11,12] and other cells including tumor cells of epithelial and nonepithelial origin.[13-15] The expression of TIMP-1 gene is growth-regulated in normal fibroblasts,[16] and can be controlled by various extracellular stimuli.[17]

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We studied on TIMP-1 immunolocalization in cultured human fibroblasts, and observed it in nuclei of some cells in addition to the cytoplasmic staining of most cells. Therefore, we further investigated the occurrence of nuclear TIMP-1 immunostaining during the cell cycle and compared it with that of proliferating cell nuclear antigen (PCNA) and 5-Bromo-2'-deoxyuridine (BrdU), labelled S-phase nuclei.

MATERIALS AND METHODS

Materials

The materials used and their sources are as follows: human gingival fibroblasts (Gin-1 cells) from Japanese Cancer Resources Bank (Tokyo, Japan); mouse monoclonal antibodies to human TIMP-1 (clones 7-6C1, 7-19F6, 7-21B12, and 7-23G9: HRP-labelled IgG Fab', which were originally raised against bovine TIMP-1 purified from the culture media of bovine dental pulp fibroblasts, were shown to crossreact with human TIMP-1.[18]) and HRP-labelled or unlabeled Fab' of normal mouse IgG from Fuji Chemical Industries Ltd. (Takaoka, Japan); FCS and RPMI medium 1640 from Gibco Co., Ltd. (Grand Island, NY, U.S.A); BrdU from Sigma Co., Ltd. (St. Louis, U.S.A); Anti-BrdU antibody from Cosmo Bio KK (Tokyo, Japan); Anti-PCNA antibody from Dako A/S (Grostrup, Denmark); and Histofine SAB-PO(M) kit from Nichirei KK (Tokyo, Japan).

Cell Culture

Gin-1 cells were grown to 40–70% confluence in RPMI medium 1640 with 10% FCS on 18 mm square glass coverslips at 37°C in 95% air and 5% CO_2. Cells under this condition were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4°C for 60 min as the control (0 h of experiment). For the experiments, the serum-containing medium was removed, and the cells were washed three times with culture medium-free PBS, and maintained in serum-free RPMI medium 1640 for an additional 48 h (growth-inhibiting culture). Then the cells were cultured in the medium containing 10% FCS (growth-stimulating culture) for up to 48 h. Cells were fixed in 4% PFA in PBS every 24 h [FCS (−) 24 h, FCS (−) 48 h, FCS (+) 24 h, and FCS (+) 48 h].

Immunostaining of TIMP-1

The direct HRP-labeled antibody method was applied. The fixed cell specimens were treated with methanol containing 0.01% hydrogen peroxide (H_2O_2) for 20 min to inhibit endogenous peroxidase activity. To block nonspecific immunoreactivity, cell specimens were treated with 100 μg/ml Fab' of normal mouse IgG in PBS for 30 min at room temperature. For immunostaining, the specimens were incubated with 30–50 μg/ml HRP-labelled Fab' of anti-TIMP-1 antibody overnight at 4°C. Of the four antibody clones which stained the cells similarly, the clone 7-6C1 was mostly used. As a negative control, HRP-labelled normal mouse IgG Fab'(30–50 μg/ml) was used instead of the specific antibodies. The specimens were dipped in 0.025% diaminobenzidine (DAB) solution containing 10 mM H_2O_2 for exactly 5 min. They were rinsed in PBS, dehydrated in a graded series of ethanol without counter staining, cleared in xylene, and mounted on a glass slide. Optical densities of immunoreactive TIMP-1 (I) of the nucleus and the cytoplasm were separately measured by a spot densitometer (PM-10 ADS, Olympus Optical Co., Ltd., Tokyo, Japan) mounted on a microscope (BH2, Olympus Optical Co., Ltd., Tokyo, Japan), with a fixed background illumination (I_0: optical density at 60 units of the meter). The spot size was 7.0 μm in diameter at ×330 magnification. The spotting area for

the nucleus was in the center and for the cytoplasm, an area with the highest density — usually the perinuclear cytoplasm — was selected (Fig. 1a).

Immunostaining of BrdU

In the BrdU labelling experiment, the medium was replaced with serum-free RPMI medium 1640 containing 0.3 μg/ml BrdU. Cells were cultured for a further 30 min and used for the experiment. Fixed cells on the cover glass were washed three times with PBS for 5 min. After denaturation of DNA by treatment with 4N HCl for 30 min, the cells were neutralized with 0.1 M $Na_2B_4O_7$ (pH 8.5) and incubated with anti-BrdU antibody (1:300 in PBS containing 0.03% Triton X-100) at 37°C for 60 min. After extensive washing with PBS, the cells were covered with FITC-conjugated rabbit anti-mouse immunoglobulin antibody (1:200 in PBS) at 37°C for 30 min. The immunostained cells were added with 50 μl of 90% glycerin in water after extensive washing with PBS, mounted on a glass slide and observed with a 490nm UV under a fluorescent microscope (BH2-RFC, Olympus Optical Co., Ltd., Tokyo, Japan). The number of nuclei with distinct fluorescence was counted at ×200 magnification, and that of negative nuclei was counted in the same field by phase contrast microscopy.

Immunostaining of PCNA

The cells on the cover glass were fixed in 4% PFA for 60 min at 4°C, and treated with 0.01% hydrogen peroxide in methanol for 30 min. After normal rabbit serum (10%) was applied for 10 min, the incubation was carried out with an anti-PCNA antibody (1:100 in PBS) at 37°C for 60 min. The specimens were then successively treated with biotinylated rabbit anti-mouse immunoglobulin antibody for 10 min and with streptoavidin for 10 min at room temperature following the instruction of the SAB-PO (M) kit. Between each incubation step, specimens were carefully washed in PBS. After DAB-reaction for 5 min, the stained cells were dehydrated, cleared and mounted on slides. Optical densities of the nucleus and the cytoplasm were separately measured by the same method as for TIMP-1.

RESULTS

TIMP-1 immunostaining was observed in the cytoplasm of a majority of Gin-1 cells and in the nucleus of some cells (Fig. 1a). The staining of the cytoplasm was usually diffuse with a higher density in the perinuclear area. The density of nuclear staining varied from cell to cell, appearing lighter or darker than the cytoplasm of the same cell. The optical densities of the nucleus and the cytoplasm were measured on a total of 400 individual cells from 4 experiments (each 100 cells), at each experimental period. Mitotic cells were round in shape and thus their cytoplasm appeared very dark, but the chromosomes were usually unstained. These cells were excluded from the measurements. Postmitotic two daughter cells usually had lightly stained nuclei. We also occasionally observed that only one of them had a densely stained nucleus (Fig. 1a, inset).

Distribution of nuclear/cytoplasmic densities of 400 individual cells at each experimental period is shown in Fig. 2. At the control 0 h, nuclear cytoplasmic densities were scattered over and below the diagonal line. At 24 h after depletion of FCS from the culture medium, both nuclear and cytoplasmic densities showed a slight decrease, and at FCS (−) 48 h the nuclear TIMP-1 densities decreased more remarkably than the cytoplasm which showed lighter nuclei (Fig. 1b). Thus the distribution of nuclear/cytoplasmic densities accumulated in the left and lower area (Fig. 2). The addition of 10% FCS to the culture medium stimulated an increase of

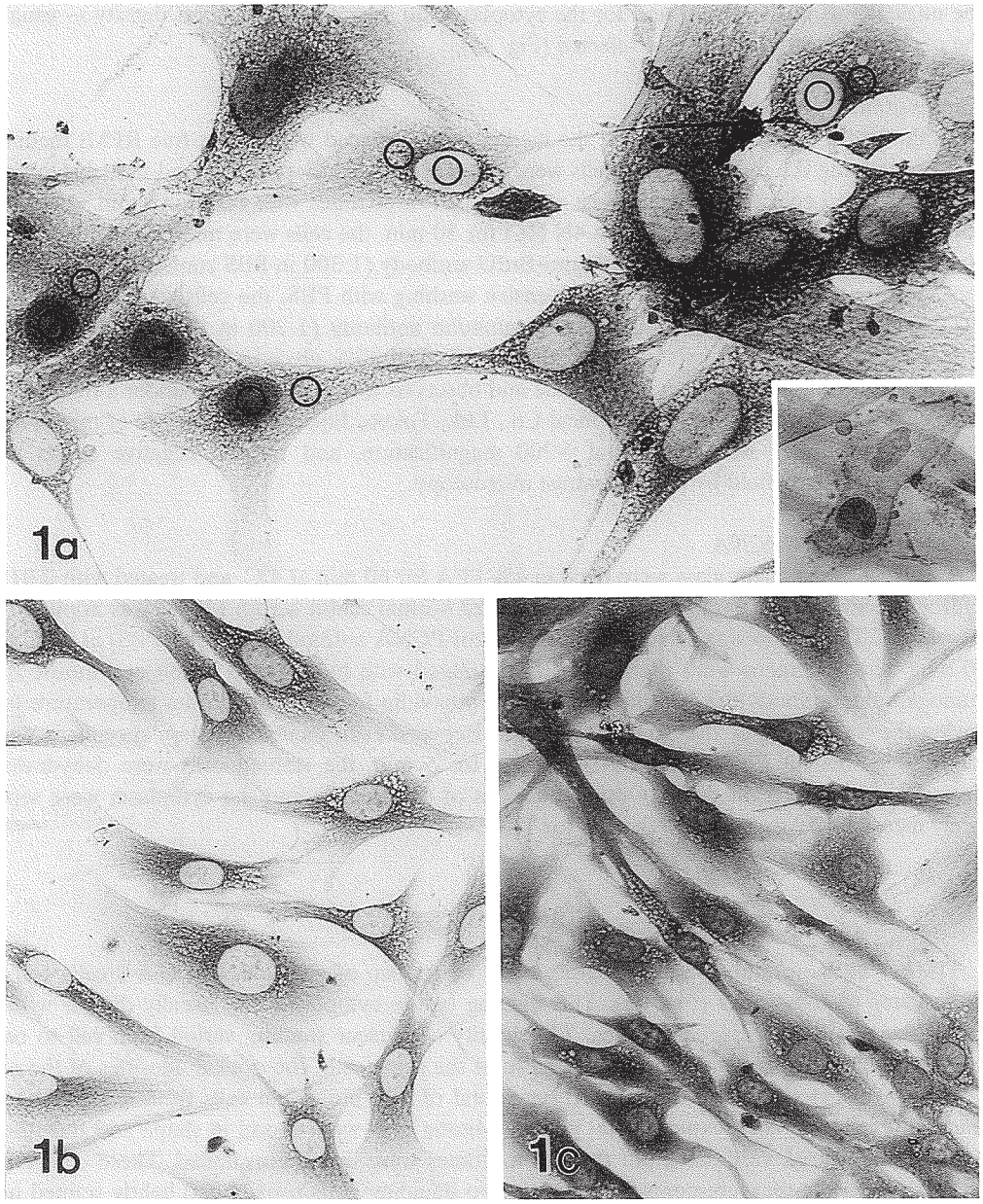

Fig. 1. Gin-1 cells stained with anti-TIMP-1 antibody (HRP-labelled IgG Fab'), without counter staining.
a: Control 0 h. The density of nuclear staining varies from cell to cell. Examples of spotted areas in the nucleus and the cytoplasm are shown by circles ×530.
Inset: one of the nuclei of postmitotic cells shows a denser staining ×330.
b: FCS (−) 48 h. The cells were cultured for 48 h in FCS-depleted medium. The nuclear density is reduced to be lighter than the cytoplasm in almost all cells ×390.
c: FCS (+) 24 h. The cells were cultured for 24 h in 10% FCS-supplemented medium after the FCS-depletion from the medium for 48 h. The nucleus is darkly stained in many cells ×380.

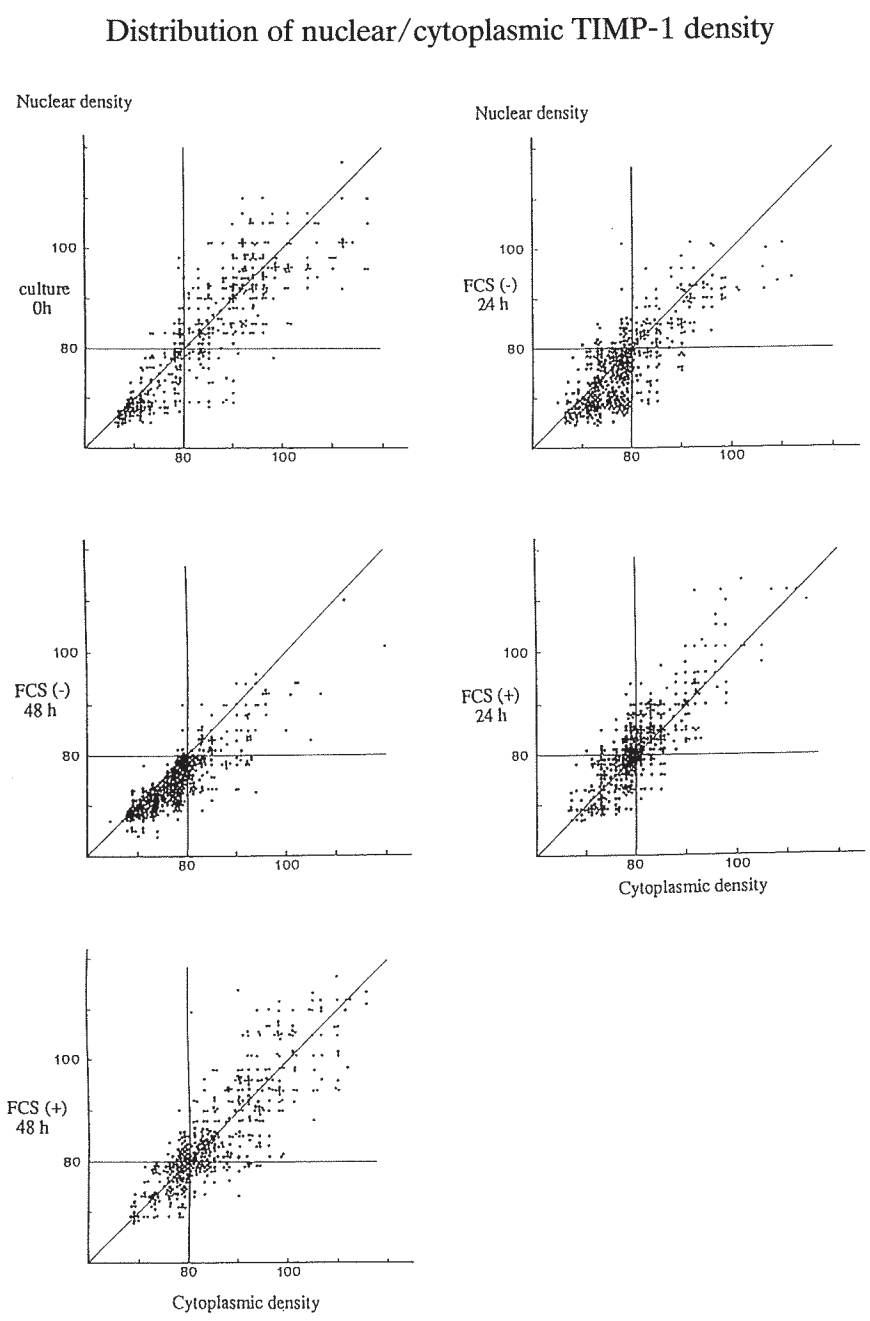

Fig. 2. Distribution of nuclear/cytoplasmic density of immunostaining of 400 individual cells from 4 cultures (each 100 cells) at each experimental period. The diagonal lines represent equal nuclear and cytoplasmic densities. Horizontal and vertical lines at 80 optical density delimitate positive and negative TIMP-1 immunostaining for the nucleus and the cytoplasm respectively. Culture 0 h: 0 h of experiment; FCS (−) 24 h and FCS (−) 48 h: cells were cultured in the FCS-depleted medium for 24 h and 48 h respectively; FCS (+) 24 h and FCS (+) 48 h: cells were cultured in the FCS-supplemented medium for 24 h and 48 h respectively, and both for 48 h in the FCS-depleted medium.

Table 1. Frequency of Nuclear or Cytoplasmic TIMP-1-positive Cells at Each Experimental Period (%)

	Control 0 h	FCS (−) 24 h	FCS (−) 48 h	FCS (+) 24 h	FCS (+) 48 h
Nuclear TIMP-1 (+)*	63.3 ± 5.2	34.5 ± 6.3	18.2 ± 2.6	51.0 ± 7.1	63.0 ± 5.3
Cytoplasmic TIMP-1 (+)*	66.3 ± 4.8	37.3 ± 3.8	36.5 ± 3.8	44.0 ± 6.3	65.5 ± 5.2

*Both nuclear and cytoplasmic immunostaining with optical densities at 80 units or higher were referred to as TIMP-1-positive.

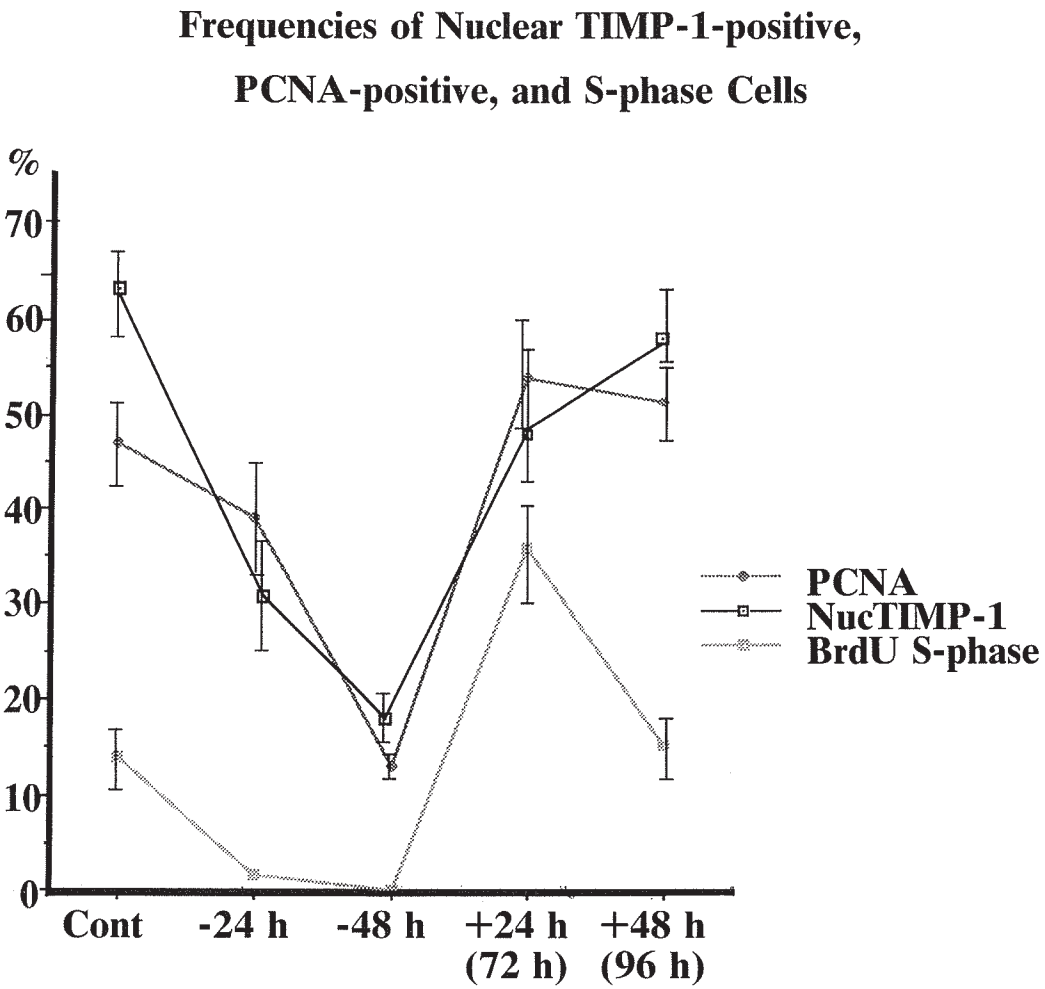

Fig. 3. A comparison of frequencies of nuclear TIMP-1-positive cells, PCNA-positive cells, and S-phase cells at each experimental period.

nuclear and cytoplasmic TIMP-1 densities at FCS (+) 24 h (Fig. 1c). The distribution of nuclear/cytoplasmic densities at FCS (+) 48 h showed a distribution similar to that of control (Fig. 2).

To quantitate immunoreactive cells, the nucleus or the cytoplasm with optical density at 80 units or higher, was arbitrarily referred to as TIMP-1-positive. Frequencies of nuclear or cytoplasmic TIMP-1-positive cells at each experimental period are shown in Table 1. Population of nuclear TIMP-1-positive cells markedly decreased to $18.2 \pm 2.6\%$ by depletion of FCS from the culture medium for 48 h, while the population of cytoplasmic TIMP-1-positive cells remained at $36.5 \pm 3.8\%$. Both recovered to the level of control at FCS (+) 48 h.

S-phase nuclei labelled with a 30 min BrdU treatment were counted in a total of 400 cells from 4 cultures (each 100 cells) for each experimental period (Fig. 3). At the control 0 h, $14 \pm 3.5\%$ of the cells were labelled. This was markedly reduced to 1.5% at FCS (−) 24 h, and no S-phase nuclei was present at FCS (−) 48 h. This result suggests that the cell growth was arrested at FCS (−) 48 h. The addition of FCS stimulated the cell growth up to $35.9 \pm 5.1\%$ of S-phase cells at FCS (+) 24 h, but the cells decreased to $15.3 \pm 3.2\%$ at FCS (+) 48 h.

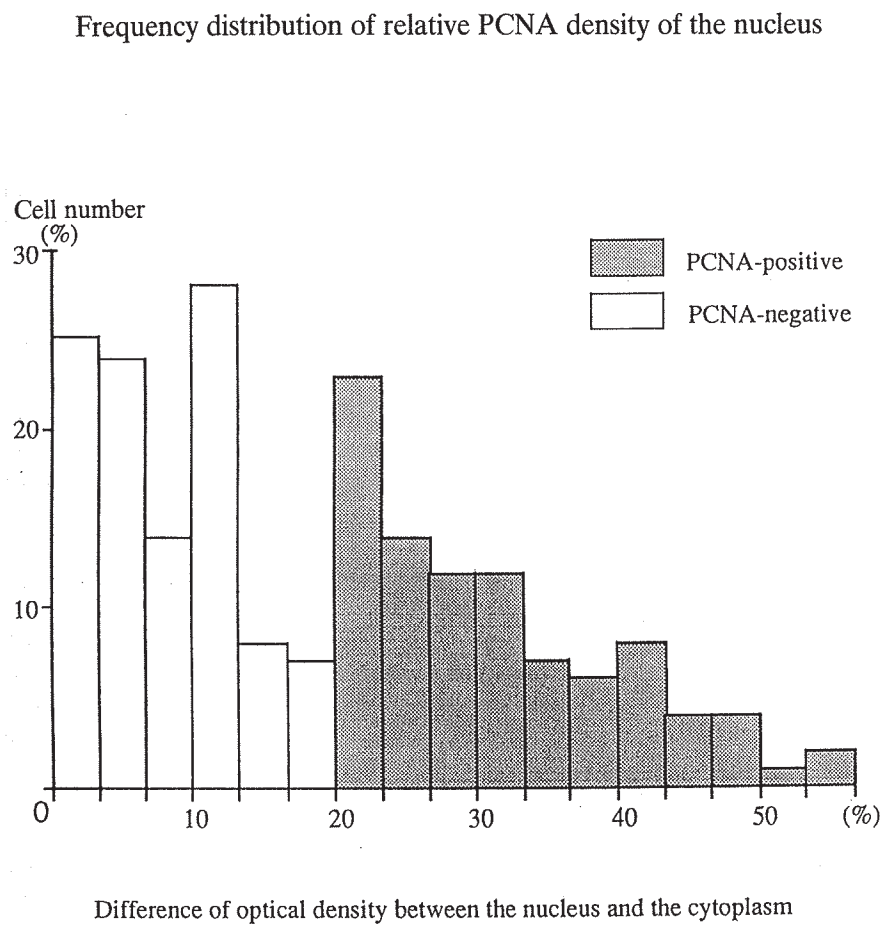

Fig. 4. Frequency distribution of relative PCNA density of the nucleus at control 0 h. Nuclei of a density 20% or higher than the cytoplasmic density are referred to as PCNA-positive (dotted columns).

PCNA-immunostaining of the nucleus varied in density, and thus it was sometimes difficult to distinguish a weak positive nucleus from negative ones. Therefore, to estimate PCNA-positive cells, the nuclear and cytoplasmic densities of the PCNA-immunostained cells were separately measured. The cytoplasm was usually unstained and the difference of optical density between the nucleus and the cytoplasm was calculated. The frequency distribution of these calculations is shown in Fig. 4. Since a distinct valley was seen below +20%, the nuclei of density 20% or higher than the cytoplasm were referred to as PCNA-positive. Frequency of PCNA-positive cells at each experimental period is shown in Fig. 3. It showed a marked decrease from $47.0 \pm 5.7\%$ (control), through $39.0 \pm 6.2\%$ at FCS (−) 24 h, to $13.0 \pm 2.3\%$ at FCS (−) 48 h, and a rapid increase up to $54.0 \pm 8.5\%$ at FCS (+) 24 h and $51.5 \pm 4.8\%$ at FCS (+) 48 h.

Frequencies of nuclear TIMP-1-positive cells, BrdU S-phase cells, and PCNA-positive cells were compared at each experimental period (Fig. 3). The frequency of nuclear TIMP-1-positive cells changed in line with that of PCNA-positive cells, but was always much higher than that of S-phase cells (Fig. 3). These results indicate that the TIMP-1-immunoreactive substance localizes in the nucleus of a population of Gin-1 cells. The nuclear TIMP-1 reactivity decreased remarkably when the cell growth was arrested, and recovered to the level of the control after stimulation of growth with 10% FCS.

DISCUSSION

The specificity of TIMP-1 immunoreactivity

The present study used HRP-conjugated Fab' of monoclonal antibodies to human TIMP-1. To block nonspecific bindings, a preincubation with Fab' of normal mouse IgG (100 μg/ml) was applied, and this completely blocked the following staining with HRP-conjugated Fab' of normal mouse IgG (35—50 μg/ml) as a negative control. After this blocking, positive staining was obtained with the HRP-conjugated Fab' of the antibodies (30—50 μg/ml).

The TIMP-1 immunostaining of the nucleus may not be the result of diffusion of HRP-reaction products from the cytoplasm, because the density of nuclear staining did not correlate with that of the cytoplasm. There is a possibility that the nuclear immunostaining represents some other substance, which is crossreactive to the monoclonal anti-TIMP-1 antibodies, and extraction of the immunoreactive substance from the nucleus and its characterization are currently under investigation in our laboratory.

Relationship between nuclear localization of TIMP-1 immunoreactivity and conditions for cell growth

In the present study, we obtained separate quantitative data of TIMP-1 immunoreactivity in the nucleus and the cytoplasm of the same cell by a spot-densitometric method. These results demonstrated that a remarkable decrease in the nuclear and cytoplasmic density occurred when the cell growth was arrested by serum depletion from the culture medium. The decrease was more prominent in the nucleus than in the cytoplasm. Furthermore, a rapid increase of nuclear TIMP-1 immunoreactivity followed serum-induced cell growth. These changes were parallel with those of PCNA in the nucleus.

PCNA is an auxiliary protein of DNA polymerase-δ,[19] which is detected in increasing quantities in the G1 phase and in decreasing quantities during the G2 phase.[20] The occurrence of TIMP-1 immunoreactivity in the nucleus may also correlate with the proliferation of the cells. A growth-factor inducible gene (16C8) of mice has been shown to be equivalent to the human TIMP-1 gene. Its transcription is induced after serum stimulation of growth-arrested mouse

fibroblasts.[16] After mitotic division, the TIMP-1 immunoreactivity in the nucleus appears to be reduced for some time. The finding of an asymmetric increase in the nuclear density of two daughter cells would indicate unsynchronization between the cell cycles.

Possible function of the intranuclear TIMP-1 immunoreactivity

Our results suggest that the TIMP-1-immunoreactive substance performs a function yet unknown within the nucleus. Although it is uncertain whether metalloproteinases, if any, are present in the nucleus, the TIMP-1-immunoreactive substance might play a role in the progression of the cell cycle by preventing unfavorable degradation of the machinery proteins in the nucleus. Another possibility may be that it positively participates, like PCNA, in the intranuclear events leading to DNA synthesis. Whether this possible function is related to the growth-promoting activity of TIMP-1, is also unknown. These postulations, however, will have to be examined further to understand the mechanisms underlying TIMP-1 localization in human fibroblasts.

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