Mitogenic Activity of S100A9 (MRP-14)

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S100A9 is a calcium binding protein found in high amounts in granulocytes and monocytes. We have shown that S100A9 stimulated the proliferation of fibroblasts, but its mechanism remains unknown. In this report, S100A9 is shown to be mitogenic and to stimulate fibroblast proliferation without other growth factors in the serum. Although an S100A8/S100A9 heteropolymer inhibited the growth of fibroblasts by chelating zinc ions, these ions had no effect on the growth-stimulating activity of S100A9. The effects of serum and S100A9 on fibroblast growth were additive, and S100A9 stimulated the growth without serum. Furthermore, S100A9 stimulated the incorporation of bromodeoxyuridine in fibroblasts. However, the effect of S100A9 on the activation of extracellular signal regulated protein kinases (ERK) was small. These results suggest that S100A9 is involved in the regulation of inflammatory processes by modulating fibroblast proliferation.

Key words calcium-binding-protein; S100-protein; mitogen; cell-growth

After incubation for 3 h at 37 °C, the plates were mixed and the absorbance at 450 nm was measured with a microplate reader model 550 (Bio-Rad Laboratories).

DNA Synthesis Assay DNA synthesis of NRK-49F cells was measured using a BrdU cell proliferation assay kit (Oncogene Research Products). NRK-49F cells were inoculated at a density of 2000 cells per well into 96-well microtiter plates in the presence of 0.5% FBS. After 24 h, the medium was removed and fresh 0.5% FBS-medium containing 300 nM S100A9 or 0.3 nM FGF-2 was added. Following 24 h stimulation, bromo-deoxyuridine was added for 6 h and its incorporation was measured as recommended by the manufacturer.

Immunoblot Analysis NRK-49F cells were incubated at a density of 40000 cells per 6 cm dish in the presence of 0.5% FBS for 24 h. Cells were stimulated with 300 nM S100A9 or 0.3 nM FGF-2 for 15—120 min. After stimulation, the medium was removed and the cells were lysed directly by the addition of sodium dodecyl sulfate (SDS) sample buffer containing 2% (w/v) SDS and 1% (v/v) 2-mercaptoethanol.

SDS-polyacrylamide gel electrophoresis was carried out using Precision Plus Protein Standards (Bio-Rad Laboratories). Proteins separated by the electrophoresis were transferred onto nitrocellulose membranes using the Mini Transblot cell (Bio-Rad Laboratories). Phosphorylated extracellular signal regulated protein kinases (ERK1/p44 and ERK2/p42) were detected using a PhosphoPlus p44/42 MAP kinase (Thr202/Thr204) antibody kit (Cell Signaling Technology) and visualized with an ECL Western blotting detection system (Amersham Biosciences) and a luminoimage analyzer (LAS-1000 plus, Fuji Photo Film). Chemiluminescence was quantitated using the Science Lab 99 Image Gauge program (Fuji Photo Film).

Statistical Analysis Data are expressed as means±standard errors of the mean. Student’s t-test was used for statistical analysis.

RESULTS

Calprotectin, a complexed form of S100A8 and S100A9, inhibited the growth of human dermal fibroblasts presumably
by the chelation of zinc ions. In contrast, we have reported that S100A9 stimulated the growth of rat kidney fibroblasts. We determined whether the zinc ions affected the growth-stimulating activity of S100A9. S100A9 stimulated the proliferation of normal rat kidney fibroblasts, NRK-49F cells; however, 10 μM ZnSO₄ had no effect on the growth-stimulating activity of S100A9 (Fig. 1). FGF-2, the positive control, stimulated NRK-49F proliferation (Figs. 1, 2). Next, the effect of serum on the growth-stimulating activity of S100A9 was examined. S100A9 stimulated the proliferation of NRK-49F about 1.5-fold regardless of the serum concentration and even in the absence of serum (Fig. 2).

The mitogenic activity of S100A9 was determined by the incorporation of bromodeoxyuridine. S100A9 and FGF-2 increased the incorporation of bromodeoxyuridine in NRK-49F cells at 24 h (Fig. 3).

NRK-49F cells were treated with 300 nM S100A9 or 0.3 nM FGF-2 for 15—120 min and immunoblots for phosphorylated ERK were performed. S100A9 induced a very weak increase in phosphorylation of ERK, when compared with the induction given by FGF-2 (Fig. 4). Quantitation of the phosphorylated ERK-1 is shown in Fig. 4.

**DISCUSSION**

In previous studies, we have demonstrated that S100A9 stimulated the proliferation of rat fibroblasts. However, Yui et al. reported that calprotectin, a complexed form of S100A8 and S100A9, inhibited the growth of human dermal fibroblasts presumably by the chelation of zinc ions. In the present study, we found that the growth-stimulating activity of S100A9 did not depend on zinc ions (Fig. 1). These facts suggested that S100A9 controlled fibroblast growth through distinct mechanisms, when it interacted with different subunits.

Although the concentration of S100A9 at the inflammatory loci was very high, a high concentration of S100A9 was required to stimulate the proliferation of fibroblasts. In ad-

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**Fig. 1.** Effect of Zinc Ion on the Growth-Stimulating Activity of S100A9

NRK-49F cells were incubated with 300 nM S100A9 or 0.3 nM FGF-2 in the presence or absence of 10 μM ZnSO₄ for 48 h. Cell numbers were measured as described in Materials and Methods. Each column represents the mean ± S.E. of 6 determinations.

**Fig. 2.** Effect of Serum on the Growth-Stimulating Activity of S100A9

NRK-49F cells were incubated with varying concentrations of FBS in the presence of 300 nM S100A9 or 0.3 nM FGF-2 for 48 h. Cell numbers were measured as described in Materials and Methods. Each column represents the mean ± S.E. of 6 determinations. Asterisks indicate significant differences from the control (p < 0.01).

**Fig. 3.** Mitogenic Activity of S100A9

NRK-49F cells were incubated with 300 nM S100A9 or 0.3 nM FGF-2 in the presence of 0.5% FBS for 24 h. Incorporation of bromodeoxyuridine was measured using BrdU cell proliferation assay kit (Oncogene Research Products). Each column represents the mean ± S.E. of 5 determinations. Asterisks indicate significant differences from the control (p < 0.01).

**Fig. 4.** Phosphorylation of ERK by S100A9

NRK-49F cells were incubated with 300 nM S100A9 or 0.3 nM FGF-2 in the presence of 0.5% FBS for 15—120 min. Phosphorylated ERK (p-ERK) and total ERK (t-ERK) were analyzed by immunoblotting and then quantitated. Phosphorylated ERK-1 that induced by S100A9 or FGF-2 was compared with p-ERK-1 (time 0 min; without stimulation) after normalization for t-ERK-1. Each point represents the mean ± S.E. of 3—6 determinations.
dition, this assay was performed in the presence of serum. Therefore, the mitogenic activity of S100A9 and the serum-depency of its activity were examined. The effects of serum and S100A9 on fibroblast growth were additive, and S100A9 stimulated the growth in the absence of serum (Fig. 2). S100A9 stimulated the incorporation of bromodeoxyuridine in fibroblasts (Fig. 3). These data suggested that S100A9 is a mitogen and its activity does not depend on the serum.

Since many growth factors activated extracellular signal regulated protein kinases (ERK), its activation by S100A9 was examined (Fig. 4). S100A9 activated ERK only scarcely. A growth factor for glial cells, S100B, which is another member of the S100 family, weakly increased the activity of ERK. These results suggested that ERK was not the main signaling pathway for the growth-stimulating activity of S100A9, but may be involved in the signaling pathway.

The present findings showed that S100A9 is a mitogen for fibroblasts. S100B and S100P stimulated the proliferation of glial cells and NIH3T3 cells, respectively. The effect of S100P on NIH3T3 cell proliferation was mediated by receptor for advanced glycation end products (RAGE). Studies to determine the receptor and signal transduction pathways leading to mitogenic activity of S100A9 are necessary.

REFERENCES