plasm and nucleus, as can be seen in both bright- and dark-field photomicrographs. These qualitative observations strongly suggest that the silver grains corresponding to the specific binding sites appear in the cytoplasm as well as the nucleus. To determine whether there was a significant difference between the distribution of silver grains obtained from total and from nonspecific binding studies, a semiquantification experiment was carried out. Results obtained from 523 cells for total binding and 502 cells for nonspecific binding, on 3 separate coverslips for each group, were analyzed. A large proportion of the totally labelled cells (89.3%) showed uniform distribution of silver grains, while 85.7% of the nonspecifically labelled cells displayed localization of the silver grains, as shown in figure 3, A–D. Furthermore, the specific binding of [^{3}H]ligand in the autoradiograms corresponded to approximately 53% of total binding.

Finally, in the cytosol of human prostates, binding sites for retinoids ($K_D = 35$ nM) have been detected[7]. Retinoid has been suggested to be a chemopreventive agent and its receptor is a member of the family of nuclear receptors[8,9]. Further studies on the relationship among S-A, retinoid and phorbol ester binding sites (i.e., receptors) are now in progress in our group.

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* To whom all correspondence should be addressed.

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Survival of BSC-1 cells through the maintenance of cell volume brought about by epidermal growth factor depends on attachment to the substratum

Y. Shiba and Y. Kanno

Department of Oral Physiology, Hiroshima University School of Dentistry, 2–3 Kasumi 1 Chome, Minami-ku, Hiroshima 734 (Japan)
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Summary. Addition of epidermal growth factor to culture medium without calf serum suppressed the increase in cell volume and then enhanced the survival of BSC-1 cells attached to culture dishes. However, these effects of epidermal growth factor were not observed in the case of cells on dishes coated with heat-denatured bovine serum albumin.
Key words. BSC-1 cells; cell survival; cell volume; cell adhesion; epidermal growth factor.

Epidermal growth factor enhances the ability of cells to survive and initiate new colonies[1,2]. Fibroblast growth factor and nerve growth factor have also been reported to promote survival of nerve cells[3–5]. Attachment of cells is required for the promotion of survival of fibroblastic 3T3-L1 cells by fibroblast growth factor[6]. Survival of BSC-1 cells derived from African green monkey kidney is also promoted by tumor-promoting phorbol ester under conditions of attachment to an appropriate substratum[7]. These reports indicate that cell adhesion is required for the promotion of cell viability. The growth factors that preferentially promote the survival of BSC-1 cells remain to be identified and it remains to be determined whether this promotion requires cell attachment. Depletion of calf serum and growth factors induces the complete disruption of cell integrity, suggesting that control of cell volume might be related to the ability of cells to survive[6,7]. Growth factors stimulate the transport of inorganic ions[8–10], and regulation of ion transport is important in sustaining cell volume[11,12]. Depletion of growth factors and other factors in the serum increases cell volume[13], so growth factors may regulate cell survival through control of cell volume.

In this study, the effect of growth factors on the survival of cultured BSC-1 cells in the absence of calf serum was investigated to clarify the involvement of the attachment of cells to the substratum, and the involvement of regulation of cell volume, in the growth factor-induced promotion of cell survival.

Materials and methods

A continuous line of kidney cells derived from the African green monkey (BSC-1), purchased from Dainip-

pon Pharmaceutical Co. (Osaka, Japan), was cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% calf serum (Gibco Laboratories, Grand Island, NY, USA). Epidermal growth factor (EGF) from mouse submaxillary glands, and fibroblast growth factor (FGF) from bovine pituitary glands, were purchased from Collaborative Research, Inc. (Lexington, MA, USA). Platelet-derived growth factor (PDGF) from procine platelets was purchased from Boehringer Mannheim GmbH (West Germany). Transforming growth factor beta (TGF-β) from human platelets was obtained from Wako Pure Chemicals (Osaka, Japan).

The cells were harvested with 0.02% EDTA dissolved in phosphate-buffered saline and 10^4 cells (about 10^3 cells/cm^2) were inoculated into DME medium without calf serum in untreated culture dishes (35 mm in diameter, Falcon No. 3001; Becton Dickinson Labware, Lincoln Park, NJ, USA) or in dishes coated with 1% heat-denatured bovine serum albumin. When the cells had been allowed to attach to the dishes for 30 min at 37 °C, the medium was changed to DME medium supplemented with calf serum, growth factors, or bovine albumin. The volumes of individual spherical cells were calculated from measurements of cell diameter made at various times after incubation. Surviving cells were inspected visually under the phase-contrast microscope at various times after the medium had been changed. Disrupted cells were scored as nonviable. Rounded-up cells appeared bright. Spread cells were scored as viable. Inspection of about 100 cells was repeated at three different places in each dish. The percentage of surviving cells was calculated from the results and expressed as the mean ± standard deviation of three observations in a typical experiment.

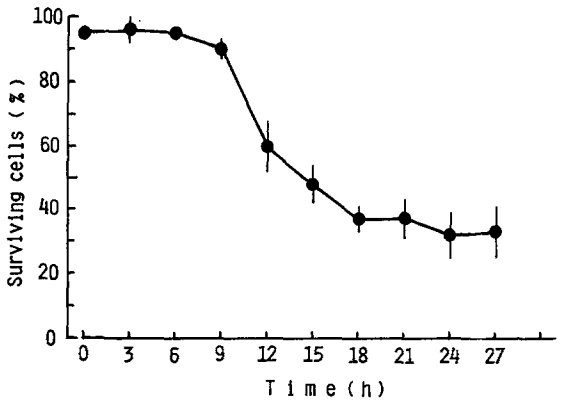

Figure 1. Time course of loss of viability of BSC-1 cells in the absence of calf serum. Each point indicates the mean ± standard deviation.

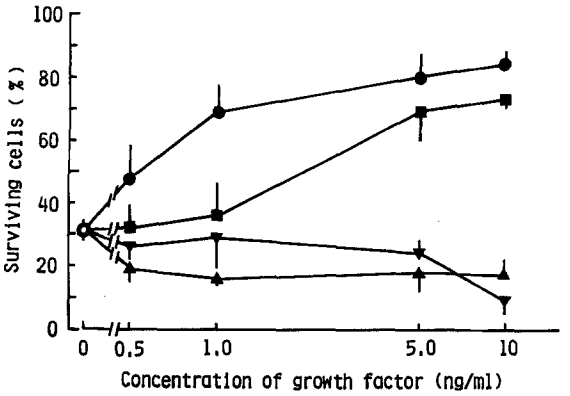

Figure 2. Effects of growth factors on survival of BSC-1 cells in the absence of calf serum. Viability was checked after a 24-h incubation of the cells on untreated dishes in DME medium supplemented with epidermal growth factor (●), fibroblast growth factor (■), transforming growth factor beta (▼) and platelet-derived growth factor (▲). Each point indicates the mean ± standard deviation.

Results

BSC-1 cells spread rapidly on untreated culture dishes after the addition of 10% calf serum. Cells were able to attach rapidly to the dishes in the absence of calf serum, but they were unable to spread. The percentage of viable cells began to decrease after a 12-h incubation in the absence of calf serum ($p < 0.005$) and reached a minimum after 24 h (fig. 1). About 70% of the cells did not maintain their integrity after a 24-h incubation in the absence of calf serum, while about 30% of the cells were able to survive and showed partially polarized spreading. The addition of epidermal growth factor at concentrations of more than 0.5 ng/ml enhanced survival of cells attached to the dishes, in a dose-dependent manner ($p < 0.05$–$p < 0.001$; fig. 2). Fibroblast growth factor at more than 5.0 ng/ml stimulated the survival of cells ($p < 0.001$). However, epidermal growth factor and fibroblast growth factor did not stimulate spreading, even though surviving cells exhibited partially spread morphology after 24 h. Platelet-derived growth factor and transforming growth factor beta were ineffective. Addition of crystalline bovine albumin at 1 mg/ml did not influence the spreading or survival of cells; the percentage of surviving cells was 26.8 ± 11.4 (mean ± standard deviation; n = 3).

The proliferation of cells that survived in the absence of calf serum or in the presence of epidermal growth factor was evaluated by assessment of the frequency of colony formation. About 100 cells, inoculated into dishes with a diameter of 35 mm, were stimulated to form colonies by addition of DME medium supplemented with 10% calf serum, after a 24-h incubation in DME medium alone or in DME medium supplemented with either calf serum or 10 ng/ml epidermal growth factor. The cells were fixed and stained with Wright's solution after 1 week in culture. When 10% calf serum was added to the cultures after a 24-h incubation without calf serum or epidermal growth factor, the frequency of colony formation was 21.2 ± 8.0% (n = 5) of that in the controls (cells incubated in the presence of 10% calf serum during the initial 24-h incubation). Addition of epidermal growth factor at

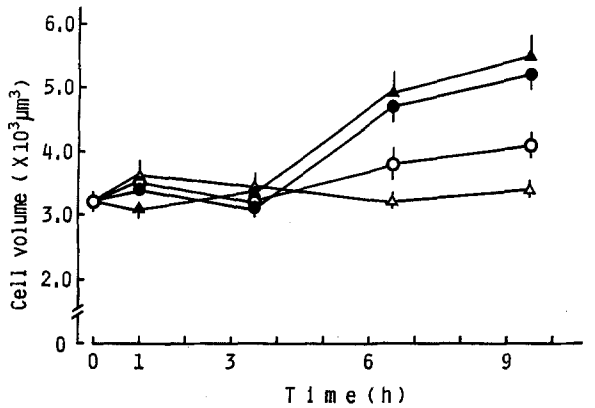

Figure 3. Effects of epidermal growth factor and attachment of cells to the substratum on the volume of BSC-1 cells. Cell volume was determined for cells cultured in the absence of epidermal growth factor on untreated dishes (○), on dishes coated with 1% heat-denatured bovine serum albumin (●), and in the presence of epidermal growth factor at 10 ng/ml on untreated dishes (△) or dishes coated with heat-denatured bovine serum albumin (▲). Each point indicates the mean ± standard error of 90 determinations of cell volume.

10 ng/ml during the initial 24-h incubation increased the frequency of colony formation to 42.1 ± 6.3% (n = 5). Coating of the dishes with heat-denatured bovine serum albumin significantly decreased the percentage of surviving cells, to 0.3 ± 0.5 (n = 3) after 24 h in the absence of calf serum, as compared to 33.6 ± 2.6 (n = 3) in the dishes without coating ($p < 0.001$). Addition of epidermal growth factor at 10 ng/ml increased the percentage of surviving cells to 76.1 ± 4.1 (n = 3) in the dishes without coating ($p < 0.001$) but, in the dishes coated with 1% heat-denatured bovine serum albumin, the percentage decreased to 2.5 ± 2.4 (n = 3), even in the presence of 10 ng/ml epidermal growth factor ($p < 0.001$).

The volume of the cells attached to the dishes began to increase 6.5 h after incubation in the absence of calf serum (fig. 3). When cells were not able to attach to the dishes coated with heat-denatured bovine serum albumin, their volume markedly increased 6.5 h after incubation in the absence of calf serum. Epidermal growth factor suppressed the increase in volume of cells attached to the dishes without coating, but it did not suppress the increase of volume of cells which had not attached to the dishes coated with heat-denatured bovine serum albumin.

Discussion

Cell survival has been studied by various methods[14, 15]. The most common method used to evaluate cell viability is the dye-exclusion test[16]. However, evaluation by the dye-exclusion test is influenced by the presence of serum, and the dye itself is toxic in the absence of serum[17]. The evaluation of the viability of rounded-up cells requires identification by some method that uses dye or radiolabeled compounds, since the morphology of viable cells is similar to that of non-viable cells. In this study, evaluation of viability of cells was carried out by inspection of cell morphology under the phase-contrast microscope; non-viable cells were completely disrupted in the absence of the serum. This evaluation in the absence of the serum gave identical results to those obtained by the dye-exclusion test and by a colorimetric assay using 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide[18]. The percentage of surviving cells was somewhat greater than the percentage of cells capable of colony formation, suggesting that almost all, but not quite all of the surviving cells, as evaluated in this study, also retained the ability to proliferate.

Epidermal growth factor markedly enhanced the survival of BSC-1 cells, in a dose-dependent manner. Fibroblast growth factor also slightly enhanced survival, but platelet-derived growth factor and transforming growth factor beta failed to enhance survival. In the case of 3T3-L1 cells, fibroblast growth factor, but not epidermal growth factor, is able to enhance the survival of cells[6]. These results suggest that epidermal or fibroblast growth factors might preferentially enhance survival, depending on whether cells are epidermal or fibroblastic in origin.

In the absence of serum, all the cells were able to attach to untreated culture dishes but they were unable to spread. Epidermal growth factor did not stimulate spreading of BSC-1 cells on the dishes, but it did enhance cell survival. Tumor-promoting phorbol ester and vitronectin promote the survival of BSC-1 cells, and this effect is associated with cell spreading[7]. Thus, there are at least two mechanisms for promotion of cell survival. One mechanism is independent of cell spreading and is mediated by epidermal growth factor, and the other is associated with cell spreading and is mediated by tumor-promoting phorbol ester and spreading factors. The adhesion of cells to an appropriate substratum itself potentiated the survival of BSC-1 cells without cell spreading, as compared to the survival of cells on culture dishes coated with heat-denatured bovine serum albumin. Epidermal growth factor was unable to enhance cell survival in the absence of cell attachment in the dishes coated with heat-denatured bovine serum albumin. Tumor-promoting phorbol ester was also ineffective under these conditions[7]. The interactions between cells and the substratum are themselves indispensable for the maintenance of cell viability in both cases.

The loss of cell viability progressed slowly in the absence of the serum. Non-viable cells were completely disrupted after a 9-h incubation in the absence of serum. Cell volume increased about 6 h after incubation in the absence of calf serum. BSC-1 cells without attachment to the dishes increased markedly in volume and then lost their viability. Epidermal growth factor suppressed the increase in cell volume only in those cells attached to the dishes, and it suppressed the loss of viability. These results suggest that the ability of cells to regulate volume

requires adhesion to an appropriate substratum, and contributes to the maintenance of cell viability. Furthermore, epidermal growth factor appears able to regulate cell volume and cell survival under conditions of cell attachment to the substratum. The enhancement of cell survival, associated with cell spreading, by tumor-promoting phorbol ester and vitronectin[7] also seems to be related to the regulation of cell volume, since stretching of the cells by spreading might lead to activation of ion flux and regulation of cell volume[19]. The volume of the cells is transiently increased in the absence of calf serum[13], and growth factors stimulate ion flux and glucose transport[8-10]. It is possible that a disturbance in ion transport may be related to the increase in volume and disruption of the integrity of cells in the absence of serum. However, further studies are necessary to clarify the mechanism of promotion of cell survival through regulation of cell volume.

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Identification of hemolytic granules isolated from human myocardial cells

Y. Kawamoto, T. Hanaichi[b], M. Naito[a] and A. Miyama

Department of Microbiology and [a] Department of Forensic Medicine, Fujita-gakuen Health University School of Medicine, Toyoake, Aichi 470-11, and [b] Central Laboratory for Electron Microscopy, Nagoya University School of Medicine, Showa-ku, Nagoya 466 (Japan)
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Summary. Human myocardial cells from fresh autopsy material contained granules which possessed hemolytic activity against guinea pig and rabbit erythrocytes. The hemolytic granules, which had a density of 1.02 and a diameter of 200–300 nm, were recovered as a microsome fraction from subcellular homogenates of human myocardial cells by differential centrifugation in 300 mM sucrose containing 0.1 mM PMSF and 10 mM EDTA. The membrane lesions caused by the granules were ring-like structures with an internal diameter of about 10–17 nm, analogous to that caused by perforin- and complement-induced lysis. However, the requirement for divalent cation differed from that for perforin-induced lysis, since the microsome-mediated lysis occurred in the presence of EDTA.
Key words. Myocardial cells; microsome granules; hemolysis; membrane lesion.

Cytotoxic T lymphocytes and natural killer lymphocytes are known to lyse a variety of target cells in the presence of calcium ions by contact-dependent mechanisms. The membrane lesions formed by large granules of these effector cells have been demonstrated as ring-like structures resembling complement-mediated transmembrane channels[1,2]. Recent studies have indicated that murine and human cytolytic T lymphocytes contain several proteins which are possibly associated with the killing of various target cells[3]. Perforin and serine protease specific for the synthetic substrate BLT are present at high levels in the large granules of cloned cytolytic T lymphocytes and are implicated in the lytic process of these effector cells[4]. Recently, regulatory mechanisms which protect the organism's own cells from cytolytic proteins have been reported[5,6]. Although perforin and C9-related