Presence of specific binding sites for [^{3}H]sarcophytol-A in cultured human skin fibroblasts

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Summary. The presence of specific binding sites for [^{3}H]sarcophytol-A in human skin fibroblasts was examined using biochemical and morphological methods. The displacement studies clearly revealed that high (K_D = 31.0 nM) and low (K_D = 6.05 μM) affinity sites were present in the intact cells. Moreover, autoradiographic studies using light microscopy revealed that the specific binding sites may exist in both the cytoplasm and the nuclei.
Key words. Sarcophytol-A; anti-promoter; specific binding sites; human skin fibroblasts; autoradiography.

Sarcophytol-A (S-A), a cembrane-type diterpene, was found in lipid extracts of the soft coral, *Sarcophyton glaucum*[1]. Very recently, it has been demonstrated that this compound possesses potent anti-tumor promoting activity in a two-stage mouse skin carcinogenesis (teleocidin as promoter) model, and also has an inhibitory effect on the hyperplasia of mouse skin, thus raising the possibility of its being used as a chemopreventive agent[2]. In an attempt to initiate the investigation of the mode of action of this agent, we have examined whether or not the specific binding sites for [^{3}H]S-A are present in human skin fibroblasts, using the methods of radioreceptor-binding assay and light microscopic autoradiography.

Materials and methods

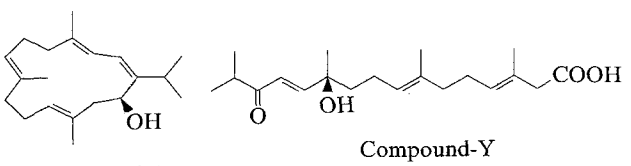
[^{3}H]S-A (sp. act. 12.2 Ci/mmol) was synthesized by the method of Kobayashi et al.[3]. The cultured human skin fibroblasts were kindly donated by Dr H. Fujiki (National Cancer Center Research Institute, Tokyo). The cells were grown at 37 °C in a humidified CO_2 (5%) incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml). For our studies, cells with passage numbers between 3 and 6 were used. Binding of [^{3}H]S-A to intact cells was assayed by the method of Horowitz et al.[4]. Briefly, the subconfluent cultures ($1.0-1.5 \times 10^5$ cells/35-mm dish) were washed twice with 2 ml of phosphate-buffered saline (PBS), and an aliquot of 2 ml of assay buffer (2 vol. of DMEM + 1 vol. of PBS + bovine serum albumin at 1 mg/ml) was added. The cell monolayer was preincubated for 60 min at 37 °C. Tritiated ligand (3 nM) was then added to the binding mixture and the binding reaction was allowed to continue for 50 min at 37 °C. When a displacer was used, the concentrations were as indicated. The monolayers were then rapidly washed four times with a total of 10 ml of ice-cold assay buffer. The cells were solubilized for 10 min at 37 °C in 2 ml of PBS containing 0.8% Triton X-100, 0.02% EDTA and 0.25% trypsin, and the radioactivity was counted in 15 ml of toluene/Triton X-100 (3:1) emulsion phosphor.

For light microscopic autoradiography, the cells were seeded in 100-mm dishes including 18-mm diameter glass coverslips for a few days. After the formation of monolayers, [^{3}H]ligand (15 nM) was added, and the cells were labelled for 50 min. Conditions of the binding reaction and determinations of the specific binding were as described above. After washing with assay buffer and water, the coverslips were immediately dried in cold air using a hair dryer. Konica NR-M2 liquid emulsion was diluted with an equal volume of water containing 0.08% sodium dioctyl sulfosuccinate at 40 °C. The coverslips were dry-mounted with this emulsion by a wire-loop method[5]. After 3–5 weeks' exposure at 5 °C, they were postfixed (methanol), developed (Konicadol-X) and stained (Giemsa solution).

Results and discussion

The specific binding of [^{3}H]S-A was defined as the difference between [^{3}H]ligand bound in the presence and absence of unlabelled S-A (50 μM), and represented 50–70% of the total binding. On addition of 3 nM [^{3}H]S-A to the binding mixture at 37 °C, specific [^{3}H]ligand binding to the intact cells occurred rapidly, reaching a maximum after about 40 min and then remaining constant for at least 90 min. This was also the case when the incubation temperature was lowered to 4 °C. However, the amount of ligand bound at equilibrium at 37 °C revealed an approximately 3.5-fold augmentation, as compared with that at 0 °C.

The stereospecificity of this [^{3}H]S-A binding was examined by the ligand displacement studies. The unlabelled S-A produced a typical biphasic displacement curve of [^{3}H]ligand binding, whereas a steep slope (Hill coefficient = 1.03) was observed when compound-Y was used (fig. 2). Compound-Y is one of the S-A analogues with a weak anti-tumor promotion activity, approximately one fifth of that of S-A[2]. Furthermore, nonlinear regression analysis[6] revealed that the inhibition curve of [^{3}H]ligand binding by unlabelled S-A best fitted a two-site model.



Sarcophytol-A Compound-Y

Figure 1. Chemical structures of sarcophytol-A and compound-Y.

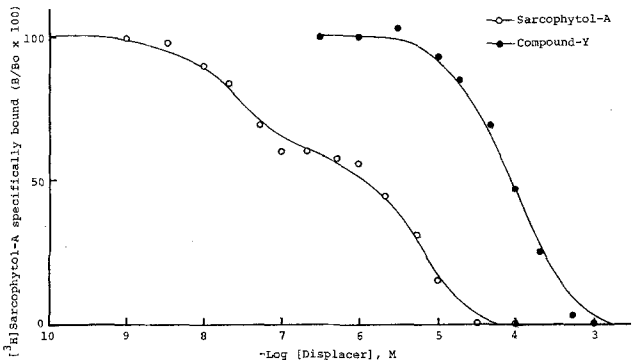


Figure 2. Competitive inhibition of [^{3}H]sarcophytol-A binding by unlabelled sarcophytol-A and compound-Y. The monolayer cells were incubated with 3 nM [^{3}H]ligand and the indicated concentrations of the binding displacer. Binding conditions and measurements of the specific binding are as described in the text. Each point represents the mean of 2–3 experiments, which varied by less than 10% between experiments. The computer-generated curves were derived from nonlinear regression analysis.

The relative distributions of the two binding sites, and their apparent K_D values, were 42.5% and 31.0 nM for the high affinity sites and 57.5% and 6.05 μM for the low affinity sites, respectively. In the case of compound-Y, the best fit was achieved with a one-site model ($K_I = 83.3$ μM). Taken together, these binding data strongly suggest the presence of specific binding sites for [^{3}H]S-A in human skin fibroblasts.

To investigate the localization of the above-mentioned specific [^{3}H]S-A binding sites, light microscopic autoradiography was performed (fig. 3). In the autoradiograms of the total binding, a halo of silver grains appeared over the cytoplasm and around the cell as well as over the nucleus. Dark-field microscopic observations of these also showed uniform distribution of silver grains over the whole cell. On the other hand, in autoradiograms of nonspecific binding almost all of the silver grains were clearly located around the cell, with a few in the cyto-

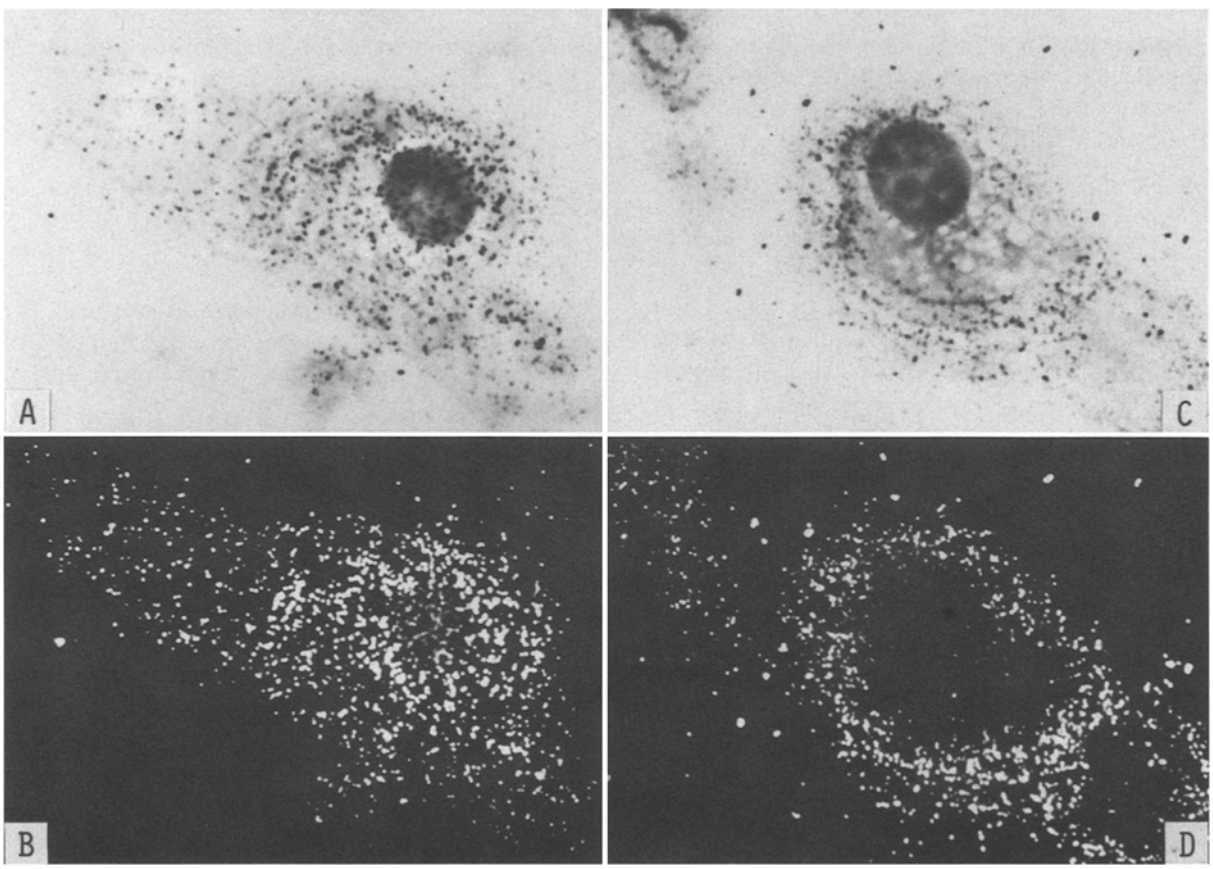


Figure 3. *A*, *B* Total binding of the [^{3}H]sarcophytol-A to the cultured cells. Uniform distribution of silver grains was seen over the entire cell in both bright-field (A) and dark-field (B) photomicrographs. *C* Bright-field and *D* dark-field photomicrographs of the cultured cell which was non-specifically labelled with [^{3}H]ligand. Many silver grains were observed around the cell, whereas there were only a few silver grains over the cytoplasmic matrix and nucleus. All magnifications ×1000.

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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1 Kobayashi, M., Nakagawa, T., and Mitsuhashi, H., Chem. pharm. Bull. *27* (1979) 2382.
2 Fujiki, H., Suganuma, M., Suguri, H., Yoshizawa, S., Takagi, K., and Kobayashi, M., J. Cancer Res. clin. Oncol. *115* (1989) 25.
3 Kobayashi, M., Kondo, K., Osabe, K., and Mitsuhashi, H., Chem. pharm. Bull. *36* (1988) 2331.
4 Horowitz, A. D., Greenebaum, E., and Weinstein, I. B., Proc. natl Acad. Sci. USA *78* (1981) 2315.
5 Miller, O. L. Jr, Stone, G. E., and Prescott, D. M., J. Cell Biol. *23* (1964) 654.
6 Yoshikawa, S., and Ishitani, R., Neuropharmacology *23* (1984) 1227.
7 Boyd, D., Beynon, L., Chisholm, G. D., and Habib, F. K., Cancer Res. *44* (1984) 5532.
8 Petkovich, M., Brand, N. J., Krust, A., and Chambon, P., Nature *330* (1987) 444.
9 Giguere, V., Ong, E. S., Segui, P., and Evans, R. M., Nature *330* (1987) 624.

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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

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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-