Modulation of Survival and Proliferation of BSC-1 Cells through Changes in Spreading Behavior Caused by the Tumor-Promoting Phorbol Ester TPA

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ABSTRACT. The effect of a tumor-promoting phorbol ester on spreading behavior was investigated to clarify the involvement of the interactions between cells and substratum in the maintenance of cell viability and the control of cell proliferation. BSC-1 cells did not spread and lost cell viability after a 24-h incubation in the absence of calf serum. Addition of calf serum initially induced radial spreading and then polarized spreading, with the formation on stress fibers and focal contact-like structure, and enhanced survival. Vitronectin also induced both radial spreading and polarized spreading, and enhanced cell survival. 12-O-Tetradecanoylphorbol-13-acetate (TPA) induced radial spreading with actin ribbons in the absence of serum. It improved the survival of cells attached to the substratum, but not in suspension. TPA suppressed polarized spreading, formation of stress fibers and of focal contact-like structure, and cell proliferation, in the presence of serum. Phorbol did not have any effect. These results suggest that enhancement of radial spreading and inhibition of polarized spreading of BSC-1 cells by TPA are closely related to the enhancement of cell survival and inhibition of cell growth.

Interactions between cells and the substratum play a crucial role in the stimulative effects of serum on survival and growth of many lines of cultured cells (5, 8, 11, 14, 26). Serum contains various factors, such as spreading factors and growth factors, that are responsible for the potentiation of survival and growth of cultured cells. Adhesion of the cells to an adequate substratum is necessary for their growth in the presence of the serum (28). In contrast, certain substrata, such as type I collagen, suppress proliferation of BSC-1 cells (a continuous line of kidney cells derived from the African green monkey), while type IV collagen promotes the survival of BSC-1 cells in the absence of calf serum (25). It remains unclear how cell adhesion modulates the proliferation and survival of cells.

Many studies using interference reflection microscopy have indicated that there are two types of adhesion of cells to the substratum (15, 16): focal contact and close contact. Focal contacts of fibroblasts with the substratum are considered to be closely associated with the functions of fibronectin and actin filaments (1, 2, 7, 10, 27). Extracellular deposits of fibronectin are observed around the focal contacts (13, 27). The close contacts seem to play a role in the regulation of cell motility (15, 16).

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However, it is unknown which type of cell adhesion is required for the survival and/or proliferation of cells.

12-O-Tetradecanoylphorbol-13-acetate (TPA), a potent tumor-promoting phorbol ester, modulates adhesiveness and growth of cultured cells (6, 21, 23, 31–33), but the details of the relationship between adhesion and growth have not been elucidated. TPA is known to disrupt focal contacts and stress fibers, which are organized during cell spreading (4, 17, 19–24). Therefore, TPA appears to be a useful tool for investigations of the possible involvement of adhesion in the survival and control of proliferation of cells.

BSC-1 cells exhibit two types of cell spreading after attachment to the substratum in the presence of calf serum: initial radial spreading and then polarized spreading (25). These two types of spreading might be related to different interactions of cells with the substratum because of adhesion of cells to the substratum and the organization of stress fibers are modulated during cell spreading. For clarification of the possible involvement of the interaction between cells and the substratum in survival and growth, these different spreading behaviors should provide a good index.

In the present study, we investigated the modulation of adhesion and organization of stress fibers by TPA to clarify their possible involvement in the regulation of survival and proliferation of BSC-1 cells.

**MATERIALS AND METHODS**

*Cells cultures.* BSC-1 cells (a continuous line of kidney cells derived from the African

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**Fig. 1.** Promotion of spreading induced by TPA in the absence of calf serum. The cells in the absence of calf serum did not spread after 2 h (a), 6 h (b), or 24 h (c). Disrupted cells (arrow) were observed 24 h later. Addition of 100 ng/ml TPA induced radial spreading (arrow) with numerous cell processes after 2 h (d) and 6 h (e), and induced polarized spreading (arrow-head) after 24 h (f). × 90
green monkey), purchased from Dainippon Pharmaceutical Co. (Osaka, Japan), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (GIBCO Laboratories, Grand Island, NY, USA). Cells in the growing phase were harvested with 0.02% EDTA dissolved in phosphate-buffered saline (PBS). They were inoculated and cultured on standard tissue-culture dishes (Corning Glass Works #25000; Corning, NY, USA) in DMEM, with or without 10% calf serum.

Chemicals. TPA and phorbol were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (such that the final amount of dimethyl sulfoxide did not exceed 0.1% by volume). Vitronectin from human plasma and fibronectin from bovine plasma were purchased from Iwaki Glass (Tokyo, Japan). Laminin from basement membrane of the Engelbreth-Holm-Swarm transplantable mouse tumor was purchased from Collaborative Research Inc. (Lexington, MA, USA). Bovine crystallized albumin and bovine serum albumin (fraction V) were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan) and from Katayama Chemical Co. (Osaka, Japan), respectively.

Determination of spreading and survival of cells. About $10^4$ cells, harvested with EDTA, were inoculated into DMEM on culture dishes (Falcon #3001). After the cells had been allowed to attach to the dishes for 30 min at 37°C, the medium was changed to DMEM supplemented with calf serum, TPA or bovine serum albumin. The cells were inspected visually under a phase-contrast microscope at various times after the medium was changed.

Spreading was scored in terms of cells in a rounded-up, radial-spreadings or polarized-spreading configuration. For determination of cell survival, disrupted cells were scored as nonviable. Rounded-up cells appeared bright, and spread cells were scored as viable. Inspect-

Fig. 2. Changes in cell morphology induced by TPA in the presence of calf serum. The cells in the presence of 10% calf serum exhibited radial spreading (arrow) after 2 h (a), and polarized spreading (arrowhead) after 6 h (b) and 24 h (c). Addition of 100 ng/ml TPA caused marked radial spreading (arrow) after 2 h (d), and sustained radial spreading (arrow) after 6 h (e). More marked spreading of cells with long irregular processes (arrowhead) was observed after 24 h (f). × 90
tion of at least 100 cells was carried out in three to five areas in each dish. The percentage of spreading cells or surviving cells was calculated from the results and expressed as the mean ± standard deviation of three to five observations in a typical experiment. Similar results were observed in at least three experiments.

Spreading and survival of cells were also monitored in 24-well plates (Corning #25820, Corning, NY, USA). The wells were coated for 12 h with one of vitronectin, fibronectin, and laminin (3 μg/well; 1.6 μg/cm²) and then for 30 min with 1% heat-denatured bovine serum albumin.

Interference-reflection microscopy. Cells cultured on glass coverslips were fixed with 3.7% formaldehyde and mounted with glycerin on glass slides. The cells were viewed under a Nikon microscope equipped with a high voltage mercury lamp. Photographs were taken on Plus-X film.

Fluorescent labeling of stress fibers. The cells were cultured on glass coverslips and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The cells were washed twice with PBS, made permeable by treatment with acetone for 5 min at −20°C, and dried. They were then stained with rhodamine phalloidin (1:100 in PBS) for 20 min and mounted with glycerin on glass slides. Stress fibers were observed under a Nikon microscope equipped with epifluorescence. Photographs were taken on Tri-X film.

RESULTS

BSC-1 cells did not spread on normal culture dishes within 6 h in the absence of

Fig. 3. Effects of TPA on stress fibers of BSC-1 cells in the absence of calf serum. Stress fibers were stained with rhodamine phalloidin. In the absence of calf serum, the cells did not spread or form stress fibers 2 h (a), 6 h (b), or 24 h (c) after inoculation. However, areas at the edges of the cell processes exhibited diffuse fluorescence (arrow). Addition of 100 ng/ml TPA induced radial spreading with actin ribbons (arrow) 2 h after inoculation (d), radial spreading with numerous processes where actin ribbons (arrow) were present after 6 h (e), and extremely long processes after 24 h (f). ×295
calf serum (Fig. 1a, b), but addition of 100 ng/ml TPA induced radial spreading with numerous irregular processes within 1 h (Fig. 1d, e). In the absence of calf serum, completely disrupted cells were observed within 24 h (Fig. 1f).

Addition of calf serum after cell attachment first induced radial spreading (Fig. 2a) and then polarized spreading 6 h later (Fig. 2b). Addition of calf serum at more than 0.5% promoted cell spreading. TPA also induced radial spreading in the presence of calf serum (Fig. 2d), while radial spreading was sustained 6 h later and polarized spreading was suppressed (Fig. 2e). TPA promoted further formation of numerous processes in the presence of calf serum after 24 h (Fig. 2f), as compared to the spreading of control cells (Fig. 2c). Phorbol at 100 ng/ml did not influence cell spreading in the absence or presence of calf serum.

Formation of actin filaments was investigated during cell spreading, using fluorescence-conjugated phalloidin. In the absence of calf serum, attached round cells extended small cell processes, and small areas at the edges of these processes exhibited diffuse fluorescence (Fig. 3a, b). Many cells were disrupted and lost viability in the absence of calf serum 24 h later, but a few bipolar spreading cells survived and did not exhibit any stress fibers (Fig. 3c). Addition of TPA induced radial spreading and increased the number of areas with diffuse fluorescence at the edges of the cells within a 1-h incubation (Fig. 3d). With time, the cells extended more processes with diffuse fluorescence (Fig. 3e), but stress fibers were not observed 24 h

Fig. 4. Effects of TPA on the formation of stress fibers, stained with rhodamine phalloidin, in BSC-1 cells cultured in the presence of calf serum. Addition of 10% calf serum induced radial spreading with fine, circular stress fibers (arrowhead) at the cell perimeter 2 h after inoculation (a), and polarized spreading with stress fibers (arrowhead) that ran longitudinally after 6 h (b) and 24 h (c). Simultaneous addition of 100 ng/ml TPA and 10% calf serum induced marked radial spreading with continuous actin ribbons (arrow) at the cell perimeter after 2 h (d). Actin ribbons (arrow) became discontinuous and were present at the edges of cellular processes after 6 h (e). Stress fibers (arrowhead) appeared but were finer and no longer straight in the presence of TPA after 24 h (f). ×295
later (Fig. 3f). Phorbol at 100 ng/ml did not alter the pattern of staining by phalloidin, which remained similar to that of the controls.

Cells whose radial spreading was induced by calf serum exhibited diffuse fluorescence due to phalloidin at their edges as well as circular, weak, filamentous fluorescence (Fig. 4a). Polarized spreading 6 h later was associated with stress fibers that ran longitudinally over the entire area of cells (Fig. 4b), and further stress fibers developed 24 h later (Fig. 4c). As a result of the addition of TPA to the medium supplemented with calf serum, radially spread cells exhibited diffuse fluorescence along their entire perimeter (Fig. 4d). With time, the diffuse fluorescence at the perimeter was restricted to the edges of some cell processes (Fig. 4e). After 24 h, irregular, fine stress fibers were formed in some polarized cells (Fig. 4f).

To examine the mode of adhesion of the cells, we observed them under an interference reflection microscope. In the absence of calf serum, no pattern of sites of cell adhesion developed (Fig. 5a, d). Addition of phorbol did not alter this configuration (Fig. 5b, e). Addition of TPA caused cell spreading and large white areas in which diffuse gray areas and small, discrete, dark areas were abundant (Fig. 5c). Dark areas disappeared 24 h later (Fig. 5f).

In the presence of calf serum, radially spreading cells exhibited gray areas (areas of close contacts) after 3 h (Fig. 6a). Phorbol did not alter these features (Fig. 6b). In the TPA-treated cells, there were numerous cell processes, in which the major areas were white, but some broad areas appeared gray and some small areas ap-

![Fig. 5. Effects of TPA on cell-to-substratum interactions, revealed by interference-reflection microscopy, in the absence of calf serum. Control cells did not form any organized structures after 3 h (a, ×860) or 24 h (d, ×860), and only diffuse gray areas and gray spots were observed. Addition of 100 ng/ml phorbol did not have any effect after 3 h (b, ×860) or 24 h (e, ×860). Addition of 100 ng/ml TPA induced cell spreading, associated with large, non-organized white areas at the perimeter of cells in which numerous, diffuse gray areas (arrow) or discrete dark areas (arrowhead) were present after 3 h (c, ×570). Numerous diffuse gray areas (arrow) were present after 24 h (f, ×570).]
peared irregularly dark (Fig. 6c). The cells cultured in the presence of serum for 24 h became reorganized in shape and exhibited numerous black spots (areas of focal contact-like structure) in the inner portions of gray areas (Fig. 6d). Phorbol did not

Fig. 6. Effects of TPA on cell-to-substratum interactions in the presence of calf serum. Control cells formed gray areas (arrow) in the presence of 10% calf serum after 3 h (a, × 860), and formed discrete, black, rod-like spots (arrowhead) after 24 h (d, × 570). Addition of phorbol had no effects on the cells after 3 h (b, × 570) or 24 h (e, × 570). Addition of 100 ng/ml TPA caused formation of diffuse gray areas (arrow) with small dark diffuse areas (arrowhead), and it caused formation of large, dis-organized white areas after 3 h (c, × 570). The formation of discrete black spots after 24 h was inhibited (f, × 570), but small, diffuse, dark areas (arrowhead) within the diffuse gray areas (arrow) were observed.

Fig. 7. Effects of cytochalasin D on spreading, cell-to-substratum interactions and stress fibers in the presence of calf serum. The cells were inoculated on standard culture dishes in DMEM with 10% calf serum and 0.5 μg/ml cytochalasin D and then incubated for 24 h. Calf serum-induced polarized spreading was inhibited (a, × 100 under the phase-contrast microscope). Addition of cytochalasin D formed only gray areas (arrow) (b, × 1200 under the interference reflection microscope) and inhibited the formation of stress fibers (c, × 480 under the fluorescence microscope).
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BSC-1 cells, harvested with EDTA solution, were inoculated in DMEM in the presence of 10% calf serum combined with 0.5 \( \times 10^{-7} \) M colchicine or 0.5 \( \mu g/ml \) cytochalasin D. The percentages of radially and polarized spread cells and of surviving cells were calculated from the results obtained after 3, 6, and 24 h, respectively, and are expressed as mean ± standard deviation of three observations. The superscript a and b indicate significant differences (P <0.05 and P <0.001, respectively) relative to the corresponding controls.

alter these features (Fig. 6e). However, treatment with TPA suppressed the appearance of these black spots while maintaining the gray areas with small, incompletely defined dark areas at the margins of the cells (Fig. 6f).

The involvement of stress fibers in cell spreading was investigated using an inhibitor of polymerization of actin, cytochalasin D. Addition of 0.5 \( \mu g/ml \) cytochalasin D in the presence of calf serum inhibited polarized spreading and formation of stress fibers and focal contacts 24 h later (Fig. 7). However, cytochalasin D did not inhibit serum-induced radial spreading (Table 1), and 76.0% of cells survived 24 h later. Colchicine did not alter spreading behavior or cell survival.

The dependence of radial spreading on the concentration of TPA in the absence of calf serum is shown in Figure 8. TPA at concentrations higher than 1.0 ng/ml promoted radial spreading within 3 h, and also promoted survival of cells after 24 h. Phorbol did not promote radial spreading or cell survival in the absence of calf serum. Addition of calf serum at concentrations higher than 0.5% caused radial or polarized spreading, and also promoted survival 24 h later.

To clarify the involvement of cell spreading in the enhancement of cell viability, the effect of TPA on survival of cells in suspension was investigated. In dishes

![Fig. 8. Effects of TPA on radial spreading and survival of BSC-1 cells in the absence of calf serum. The cells harvested with EDTA solution were inoculated into standard culture dishes in DMEM with the indicated concentrations of TPA. The percentages of radially spread cells (○) and of surviving cells (△) were determined after 3 h and 24 h, respectively. The effects of phorbol are indicated by (•) and (○), respectively. Each point represents the mean ± standard deviation from three observations.](image-url)
coated with heat-denatured bovine serum albumin (3 mg/well; 1.6 mg/cm²), addition of 100 ng/ml TPA completely failed to induce attachment and spreading after a 1-h incubation in the absence of calf serum. Moreover, TPA failed to promote cell survival 24 h later. The percentage of surviving cells on the dishes coated with heat-denatured bovine serum albumin was 15.6 ± 8.9 (mean ± standard deviation, n = 3) in the presence of 100 ng/ml TPA, while it was 88.2 ± 7.7 (n = 3) in case of the standard dish (P < 0.001).

Furthermore, the effects of spreading factors on cell survival were investigated to strengthen the relationship between cell spreading and cell survival. Coating of the dishes with vitronectin, but not with fibronectin or with laminin, induced both radial spreading and polarized spreading in the absence of calf serum (Table 2). Vitronectin also promoted survival of cells. The survival of cells unattached to the dishes coated with heat-denatured bovine serum albumin was reduced as compared to the survival on the standard non-coated dishes.

TABLE 2. EFFECTS OF COATING OF CULTURE DISHES WITH CELL-SPREADING FACTORS ON SPREADING AND SURVIVAL OF BSC-1 CELLS IN THE ABSENCE OF CALF SERUM.

<table>
<thead>
<tr>
<th></th>
<th>% Radially spread cells</th>
<th>% Polarized spread cells</th>
<th>% Surviving cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3.1 ± 0.5</td>
<td>14.7 ± 6.1</td>
<td>33.6 ± 2.6</td>
</tr>
<tr>
<td>BSA</td>
<td>0 ± 0*</td>
<td>0 ± 0*</td>
<td>0.3 ± 0.5*</td>
</tr>
<tr>
<td>VN</td>
<td>23.2 ± 1.8*</td>
<td>40.7 ± 7.3*</td>
<td>69.4 ± 2.9*</td>
</tr>
<tr>
<td>FN</td>
<td>4.2 ± 0.9</td>
<td>8.8 ± 4.7</td>
<td>28.9 ± 7.7</td>
</tr>
<tr>
<td>LN</td>
<td>0.6 ± 0.6*</td>
<td>2.8 ± 1.4*</td>
<td>25.8 ± 8.6</td>
</tr>
</tbody>
</table>

BSC-1 cells, harvested with EDTA solution, were inoculated in DMEM in the absence of calf serum into standard dishes (N) or dishes coated with 3 mg/well (1.6 mg/cm²) heat-denatured bovine serum albumin (BSA), and 3 µg/well (1.6 µg/cm²) vitronectin (VN), fibronectin (FN), or laminin (LN). The percentages of radially spread cells, polarized spread cells and surviving cells (mean ± standard deviation of three observations) were calculated from results obtained 3, 6, and 24 h after the initiation of incubation, respectively. The superscript *a, b, c and d indicate significant differences (P < 0.05, P < 0.02, P < 0.01 and P < 0.001) relative to the corresponding controls.

Fig. 9. Effects of TPA on growth of BSC-1 cells in the presence of calf serum. The cells were cultured for 5 days in the presence of 10% calf serum with TPA at the concentrations indicated. The inhibitory effect of TPA on growth is indicated as the percentage inhibition of control growth. Each point represents the mean ± standard deviation from three experiments.
The effect of TPA-induced inhibition of polarized spreading on cell growth was investigated to clarify the relationship between cell spreading and cell growth. Addition of TPA in the presence of calf serum inhibited cell proliferation, although addition of phorbol did not. The dose-dependency of growth inhibition is shown in Figure 9. Inhibition of growth by TPA was observed at the concentrations greater than 1.0 ng/ml and reached a maximum at 50 ng/ml.

**DISCUSSION**

Spreading of BSC-1 cells in the presence of calf serum was divided into two phases: the first phase involved radial spreading and the subsequent phase involved polarized spreading. Radial spreading was associated with close contact with the substratum and circular stress fibers. Polarized spreading was associated with formation of longitudinal stress fibers and focal contact-like structure which was less developed than that of fibroblastic cells (24). These results are consistent with the observation that cytochalasin D inhibited polarized spreading in addition to suppressing the formation of focal contact-like structure and longitudinal stress fibers.

Various types of spreading factors have been identified in the serum (9, 12, 30). Vitronectin, alone among the various spreading factors tested, induced radial spreading of BSC-1 cells and subsequent polarized spreading. Fibronectin and laminin did not induce radial or polarized spreading. Thus it appears that vitronectin is one factor in calf serum which is responsible for the radial or polarized spreading of BSC-1 cells.

TPA was able to induce radial spreading of BSC-1 cells in the absence of calf serum, but it inhibited subsequent polarized spreading even in the presence of calf serum. TPA-induced radial spreading was not associated with the formation of focal contact-like structure or stress fibers, but it was associated with the formation of actin ribbons at the periphery of cells. The effects of TPA on stress fibers and focal contacts have been observed in various types of cell (4, 19-24).

Cells whose radial spreading was induced by TPA maintained their viability even in the absence of calf serum, while rounded-up cells did not maintain their viability in the presence of TPA. TPA did not promote the survival of cells without attachment of the cells to the dishes coated with heat-denatured bovine serum albumin. This result suggests that radial spreading behavior is indispensable for the promotion of survival of the cells. This hypothesis is also strengthened by the results that vitronectin induced radial spreading and promoted survival of the cells in the absence of calf serum and that most cells treated with cytochalasin D failed to spread with polarized morphology but were viable.

However, cell attachment to the standard dishes without spreading also slightly increased the survival of cells in the absence of calf serum. This is consistent with the result that coating the dishes with fibronectin or laminin also slightly potentiated cell survival. When the cells were unable to attach to the substratum such as heat-denatured bovine serum albumin, very few cells survived. These results suggest that non-specific attachment of cells to the substratum also slightly enhances the survival of BSC-1 cells.

Addition of calf serum induced polarized spreading and proliferation of cells. TPA inhibited polarized spreading and the proliferation of BSC-1 cells in the presence of calf serum. In addition to inhibiting polarized spreading, cytochalasin D also inhibited proliferation of cultured cells (3, 18, 29). It should be emphasized
that polarized spreading with the formation of stress fibers and focal contact-like structure is closely related to the serum-induced proliferation. Type I collagen as substratum also suppresses proliferation of cells and formation of well-developed stress fibers, but cells treated with type I collagen are long and bipolar in shape, with partial spreading (25). Serum-induced proliferation of cells might require an appropriate interaction of cells with the substratum to cause the organization of stress fibers and focal contact.

The effects of TPA on cell proliferation depend on the cells treated; TPA can exert a stimulative (21, 24, 31) or inhibitory effect (33). Focal contacts and stress fibers are disrupted by TPA, independent of the effect of TPA on proliferation. These effects can probably be explained by the fact that the modulation of the interactions between cells and the substratum plays a crucial role in the regulation of cell proliferation, while subsequent signals linked to their modulation might exert an opposing effect, depending on the cells in question.

In summary, the ability of the cells to survive and proliferate is closely related to their spreading behavior, which is regulated by the interactions between cells and the substratum. However, further studies are required to clarify how spreading behavior regulates survival and proliferation of BSC-1 cells.

REFERENCES


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