Rous sarcoma virus-transformed cells develop peculiar adhesive structures along the cell periphery

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SUMMARY
Alteration of the cell/substratum adhesive structures of rat fibroblasts (3Y1 cells) upon transformation by Rous sarcoma virus (RSV) was investigated by immunofluorescence microscopy. In serum-containing culture medium, 3Y1 cells developed focal adhesions as their main adhesive structures, while BY1 cells expressed peculiar close contacts along the cell periphery with the vitronectin receptor integrin, in addition to podosomes. These peripheral close contacts are referred to as the peripheral adhesions. The peripheral adhesions were observed as a darker region than podosomes by interference reflection microscopy. They were more easily destroyed by incubating the cells with RGD-containing peptide than were the focal adhesions. In contrast to focal adhesions and podosomes, actin bundles were not detected within the peripheral adhesions, where pp60v-src and tyrosine-phosphorylated proteins accumulated. Expression of the integrin was determined by the substratum composition when BY1 cells were cultured in serum-free culture medium. Under such conditions, BY1 cells expressed the peripheral adhesions within 3 hours on adhesion molecule-coated glass. On the other hand, in serum-containing medium, they first developed focal adhesions transiently at their early stage of adhesion, and then the peripheral adhesions were predominantly expressed within 12 hours. Podosomes were formed in a time course similar to that of the peripheral adhesions. These findings suggest that the peripheral adhesion is a class of stable adhesive structure distinct from the focal adhesion or podosome of BY1 cells. Similar close contact-type peripheral adhesions with the integrin were also observed in a variety of cultured cells such as normal fibroblasts at their logarithmic growth phase, phorbol ester-treated fibroblasts, and several malignant tumor cells, with poorly organized focal adhesions and stress fibers. These findings further suggest that the peripheral adhesions may be widely involved in the adhesion of cells that inadequately develop stress fibers and focal adhesions.

Key words: Rous sarcoma virus, adhesive structure, close contact, integrin, vitronectin, fibronectin, actin, podosome, focal adhesion, src, confocal laser scan microscopy

INTRODUCTION
Malignant transformation by tumor virus causes complex changes in cell shape, cytoskeletal organization and cell adhesiveness to the substratum (Burridge, 1986), all of which have been regarded as closely related to the altered expression of the extracellular matrix (ECM) and the ECM receptors on the cell surface (Turner et al., 1991). Such changes on the cell surface may cause the alteration of the cell/substratum adhesive structures. These adhesive structures have been classified into two different types, ‘focal contacts’ and ‘close contacts,’ according to the distance between the ventral plasma membrane and the substratum, shown by interference reflection microscopy (IRM) (Izzard and Lochner, 1976; Kolega et al., 1982). Focal contacts are small discrete areas with close proximity to the substratum (10-15 nm), characterized by black IRM images. These contacts have been regarded as the regions where thick actin bundles (stress fibers) terminate, and are also called ‘focal adhesions’ (Burridge et al., 1988). Close contacts are at a distance of approximately 30-50 nm from the substratum and are characterized by variable gray IRM images.

Accompanying transformation by RSV, focal contacts characteristically diminish in number and size, while close contacts flourish (Tarone et al., 1985). Among the close contacts, the cell surface protrusion, called the podosome (Tarone et al., 1985), or rosette (David-Pfeuty and Singer, 1980), has been regarded as the key structure in the invasion of RSV-transformed cells with highly dynamic characteristics (Burridge et al., 1988, Chen, 1989). However, the precise role of close contacts other than podosomes has not been well characterized.

The integrins, a family of cell-surface proteins mediating cell-substratum and cell-cell interactions, may be involved in many cellular events (Hynes, 1992), and also in invasive cascades (Ruoslhti, 1989). They are het-
Cells

Human osteosarcoma MG-63 cells were obtained from Takara (Japan). Polyclonal antibodies against phosphotyrosine (6D-12) were obtained from MBL (Japan), rhodamine-phalloidin, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, and rhodamine or FITC-conjugated horse anti-mouse IgG from Sigma.

**MATERIALS AND METHODS**

**Cells**

BY1 is a clonal cell line of RSV-transformed 3Y1 cells derived from Fisher rat embryos (Kimura et al., 1975). These cell lines and NRK (normal rat kidney) cells were generous gifts from Dr R. Hirai (Tokyo Metropolitan Institute of Medical Science). Human osteosarcoma MG-63 cells were obtained from Takara (Japan) and human epidermal carcinoma KB cells were purchased from the American Type Culture Collection (USA). All the cells were seeded on glass coverslips and cultured for 18 to 48 hours at 37°C with 5% CO₂ and 100% humidity in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), except for NRK cells (5% FCS). When cultured in serum-free DMEM, the glass cover slips were coated with 0.1-0.5 µg/ml of bovine plasma vitronectin (VN) (Funakoshi, Japan) in phosphate buffered saline (PBS) or human plasma fibronectin (FN) (prepared in our laboratory by the modified method of Englert and Russlaiti, 1977) in carbonate/bicarbonate buffer. Phorbol ester treatment was performed on NRK cells as previously reported (Sobue et al., 1988).

**Immunoreagents**

Polyclonal antibodies against human FNR and VNR were purchased from Telios (USA). Monoclonal antibodies against pp60 vinc (mAb 327) and chicken gizzard vinculin (VIN-11-5) were purchased from Oncogene Science, and Sigma (USA), respectively. Monoclonal antibody against phosphotyrosine (6D-12) was obtained from MBL (Japan), rhodamine-phalloidin, fluoro-}

**Gel electrophoresis and immunoblotting**

Cell lysates were boiled for 5 minutes in a sample solution containing 2% sodium dodecyl sulfate (SDS), 30 µM 4-aminophenyl methanesulfonyl fluoride (pAPMSF), 10 µg/ml leupeptin and 0.05 mM disopropyl fluorophosphate (DFP) without 2-mercaptoethanol (2ME) (Laemmli, 1970). Protein concentrations in cell lysates were adjusted to 0.35 mg/ml using the BCA protein assay system (Pierce, USA). Proteins in cell lysates (approximately 7-12 µg/lane) were separated by SDS-polyacrylamide gel electrophoresis, and subsequently transferred to nitrocellulose sheets (Towbin et al., 1979). The sheets immunoblotted by the primary antibodies were incubated with anti-rabbit goat or anti-mouse horse IgG labeled with alkaline phosphatase (Promega, USA).

**Immunolabeling of cells and microscopic observation**

The proteins except for F-actin were localized by indirect immunofluorescence microscopy. The cells grown on coverslips were fixed with 3.5% paraformaldehyde in PBS for 15 minutes at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 3 minutes. After blocking with 1% bovine serum albumin (BSA) in PBS for 10-30 minutes, the cells were incubated with the primary antibody diluted in PBS containing 1% BSA and 0.02% Triton X-100 for 30 minutes at 37°C, and then rinsed with PBS and immunoreacted with the FITC- or rhodamin-labeled goat anti-rabbit IgG or horse anti-mouse IgG. For double labeling of both F-actin and the protein, the secondary antibody solution included rhodamine-phalloidin. After extensive washing with PBS, the coverslips were mounted and viewed using a Zeiss fluorescence microscope equipped with interference reflection microscopy (IRM) optics (Izzard and Lochner, 1976). Normal rabbit or mouse IgG were used for the control experiment instead of the respective antibodies.

Fluorescence photographs were recorded with Kodak Tri-X films and IRM pictures with Kodak T-max films, and were developed in Kodak D-76.

**Confocal laser scan microscopy**

The cells immunostained with the anti-VNR antibody, and labeled with rhodamine-phalloidin as described above were also observed in a confocal laser scan microscope (CLSM; Leica Lasertechnik GmbH, Germany). An argon ion laser operating at 488 nm or 514 nm was used as the excitation source. The scan time was 0.25 seconds per frame. Series of 8 frames with a depth of 0.5 µm each in Z-axis from the base to the dorsal side of the fixed cells were examined. All images were photographed with Fuji Fujichrome 100 film.

**GRGDSP treatment**

The synthetic peptide GRGDSP derived from the cell-binding domain of FN and also VN (Smith and Cheresh, 1988), and its analogue GRGESP were purchased from Iwaki glass (Japan). The cells had been cultured for 2 days, then the culture medium was removed and replaced with the same serum-containing medium with 200 µg/ml of the GRGDSP peptide, or the GRGESP peptide as a control. At 5 minutes after the addition of the peptide, the cells were fixed and subjected to the immunolocalization study.

**Time-dependent changes of podosome, focal adhesion, and the peripheral adhesion formation**

BY1 cells were fixed at 1, 3, 6, 12, 24 and 36 hours after seed-
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ing on glass, and the localization of the integrin and F-actin were examined together with the corresponding IRM image. Due to the presence of short F-actin bundles, podosomes were easily able to be recognized as rhodamine-fluorescent 'dots' distributed on the ventral membrane of the cell. The ratio of the number of podosome-bearing cells to that of all the cells was counted on the micrographs, and was regarded as the ratio of podosome formation. Judging from the distribution of the integrin, actin, and the IRM images, focal adhesion- or peripheral adhesion-bearing cells were identified. The ratio of the formation of each structure was calculated in the same way as for the podosomes. In each experiment, 150 cells were counted. The data were obtained from three separate experiments.

RESULTS

General features of the cell/substratum adhesive structures in normal fibroblasts and their RSV-transformed cells

The present study was performed on the cell/substratum adhesive structures of a clonal cell line of rat fibroblast (3Y1) and its RSV-transformed counterpart (BY1) grown on glass coverslips for 2 days. 3Y1 cells displayed a well-spread fibroblastic morphology and formed focal adhesions identified by IRM as an arrowhead-like black light intensity. Stress fibers terminated at focal adhesions (Fig. 1A,B, indicated by arrowheads). By contrast, BY1 cells displayed a round or spindle-shaped morphology and short F-actin aggregates were found within podosomes. In the presence of serum, most podosomes were localized at the center area of the ventral membrane as previously reported (Chen et al., 1986). The IRM images of podosomes differed from each other, though most of them showed pale gray images (Fig. 1C,D), indicating a variation in the distance between podosomes and the substratum. Close contacts were distributed along the cell periphery, where transverse actin filaments were observed (Fig. 1C,D arrowhead). In most cells, close contacts at the cell periphery were darker than podosomes, as seen by IRM (Fig. 1C,D). Less than 20% of BY1 cells adhered to the substratum mainly via podosomes without forming the peripheral close contacts (data not shown).

Expression and distributional changes of the integrins upon RSV-transformation

To examine the expression and distribution of the FNR and the VNR accompanying RSV-transformation, an immunoblotting study was performed on the cell lysates of 3Y1 and BY1 cells with the anti-FNR, and the anti-VNR polyclonal antibodies. These antibodies specifically cross-reacted with the β chain of FNR (approximately 110 kDa) or VNR (approximately 95 kDa) under non-reducing conditions. As previously reported (Plantefaber and Hynes, 1989), expression of the FNR decreased accompanying transformation (Fig. 2A,B), while that of the VNR was preserved (Fig. 2C,D).

Localization of the FNR and the VNR was investigated in normal and transformed cells cultured on glass coverslips for 2 days in the presence of serum. In 3Y1 cells, FNR

Fig. 1. Distribution of F-actin in normal fibroblast cells (3Y1; A) and in RSV-transformed cells (BY1; C). The cells were fluorescently labeled with rhodamine-phalloidin. The same field of the cells was also observed by interference reflection microscopy (IRM) to recognize cell/substratum adhesion areas (B,D). 3Y1 cells form focal adhesions (B, arrowheads) in which thick actin bundles (stress fibers) terminate (A, arrowheads). In round BY1 cells, dot-like aggregates of F-actin are observed within podosomes at the center of the ventral plasma membrane (C). Belt-like close contacts are located along the cell periphery as major adhesive site to the substratum (D, arrowhead). Note that some transverse actin bundles are observed near the peripheral close contacts (C,D arrowhead). Bars, 30 μm.
labeling was diffusely distributed throughout the ventral membrane, while some labeling was enriched in focal adhesions (Fig. 3A,B arrowheads) as previously reported (Chen et al., 1986). On the other hand, VNR labeling was preferentially concentrated in focal adhesions (Fig. 3C,D). In BY1 cells, FNR labeling was diffusely distributed, but not specifically concentrated in peripheral close contacts identified by IRM imaging (Fig. 4A,B). VNR labeling in BY1 cells was preferentially detected in peripheral close contacts (Fig. 4C,D), while diffuse staining was also observed in the cytoplasm. We refer to these peripheral close contacts with the integrin (VNR) as the peripheral adhesions.

We further investigated the effect of synthetic peptide GRGDSP, which is derived from the essential sequence of a cell-binding domain of FN and VN, on the preformed peripheral adhesions. When BY1 cells were incubated with GRGESP peptide as a control, both the peripheral adhesions identified by VNR labeling and IRM imaging (Fig. 5A,E) and podosomes detected by F-actin aggregates and IRM image (Fig. 5C,E) remained intact. In the GRGDSP-treated cells, by contrast, most of the peripheral adhesions were destroyed and VNR labeling on the ventral plasma membrane was lost except for some dim punctate staining (Fig. 5B,F, arrows), and some cells detached from the sub-stratum. GRGDSP treatment was also performed on 3Y1

![Fig. 2. Immunoblotting showing the crossreactivity of the antibodies against the fibronectin receptor (FNR) and the vitronectin receptor (VNR) under non-reducing conditions. Lanes A,C and B,D indicate the cell lysates of 3Y1 and BY1 cells, respectively; 8 µg of protein was applied to each lane. The concentration of acrylamide in SDS-PAGE was 7.5%. The arrowheads indicate molecular masses of 205 (1), 116.5 (2), and 80 kDa (3), respectively. Each antibody specifically crossreacted with the β-chain of the respective integrin. Note that expression of the FNR is remarkably reduced after transformation (A and B), while that of the VNR is preserved (C and D).]

![Fig. 3. Localization of the FNR (A) and the VNR (C) in 3Y1 cells was examined with the antibodies against the respective proteins. (B and D) The IRM pictures corresponding to the cells on the left side. FNR labeling is observed throughout the ventral membrane of 3Y1 cells including focal adhesions (A,B, arrowheads), while the VNR is preferentially localized in focal adhesions (C,D). Bar, 30 µm.](image)
cells, when focal adhesions with the termination of actin bundles (stress fibers) was, contrary to the peripheral adhesions, almost preserved (data not shown) as previously reported (Chen et al., 1986).

**Localization of the VNR and actin-bundles in BY1 cells by CLSM**

To examine the relationship between the VNR and actin filaments in podosomes and the peripheral adhesions of BY1 cells, localization of the VNR and actin filaments was analyzed using confocal laser scanning microscopy (Fig. 6). Fluorescence of the VNR cluster (indicated by green fluorescence) could be detected from the first slice in the peripheral adhesions (large arrow) and some tips of podosomes (arrow), while actin bundles (orange fluorescence) were scarcely observed in the first and second slices. Transverse actin bundles just above the peripheral adhesions (slice E, large arrow) were 3-4 slices (1.5-2 µm) more dorsally located than the VNR cluster in the peripheral adhesions (slices A and B, large arrow). By contrast, fluorescence of the VNR and actin bundles were detected in the same slice in focal adhesions of 3Y1 cells, indicating that the VNR and actin bundles shared the same distance from the substratum (data not shown). The VNR in the peripheral adhesions, therefore, does not seem to contain actin bundles.

The actin fluorescence within podosomes was most remarkable in slices D and E. Faint fluorescence of the VNR was observed in most podosomes, while the fluorescence was brightly detected in some podosomes that adhered proximately to the substratum (slices A and B, arrow).

**pp60^v-src and tyrosine-phosphorylated proteins accumulate in the peripheral adhesion**

In RSV-transformed cells, localization of pp60^v-src in podosomes and residual focal adhesions has been reported (Jove and Hanafusa, 1987; David-Pleuty and Nouvian-Dooghe, 1990; Mueller et al., 1992). In BY1 cells, not only podosomes (Fig. 7A,B,C, arrows) but also the peripheral adhesions (Fig. 7A,C, arrowheads) contained pp60^v-src. Tyrosine-phosphorylated proteins were also distributed at the tips of podosomes (Fig. 7A,C, arrowheads) and in the peripheral adhesions (Fig. 7D,F, arrowheads). On the other hand, tyrosine-phosphorylated proteins were not clearly detected in focal adhesions of 3Y1 cells unless the cells were incubated with 10 mM vanadate before fixation (data not shown).

**Expression of the integrins in the peripheral adhesions depends on the substratum composition**

A variety of growth factors and adhesion molecules in serum profoundly affect the formation of cell/substratum adhesive structures. In order to exclude the effects of serum, BY1 cells were cultured on glass coverslips coated with purified plasma VN in serum-free medium. Under these conditions, the cells adhered and flattened rapidly, and
Podosomes could be observed at the early stage of adhesion, i.e. in 37% of the cells in 1 hour, and 72% in 3 hours (Fig. 8). At 1 hour after plating, 77% of the cells formed broad, grey close contact along the cell periphery (Fig. 8, Fig. 9C, arrowheads), where the VNR cluster was found to be scarce (Fig. 9B,C, arrowheads). Podosomes were observed in the center of the ventral plasma membrane with the VNR cluster (Fig. 9A,B,C, arrows). At this period, the peripheral adhesions possessing the VNR cluster were observed in only 26% of the cells (Fig. 8). In 3 hours, 70% of the cells developed the peripheral adhesions as their main cell/substratum adhesive structure, in which the VNR preferentially clustered (Fig. 8, Fig. 9E,F, arrowheads). Even at this point, no focal adhesions were observed. The peripheral adhesions with the FNR cluster were also observed when BY1 cells were cultured for 3 hours on coverslips coated with FN (Fig. 9H,I, arrowheads). In most cells, however, the FNR cluster was observed only in some part of peripheral close contacts (compare Fig. 9H and I).

**Time-dependent changes of BY1 cell adhesive structures in the presence of serum**

A kinetic analysis of adhesive structure formation in BY1 cells was performed in the presence of serum (Fig. 10). In the first hour of culture, most cells remained small and round, and adhered to the substratum by diffuse close contacts, and no specific VNR cluster was observed on the ventral plasma membrane (data not shown). In 3 hours, cells forming focal adhesions (Fig. 11A,B,C, arrowhead) became conspicuous (approximately 30%, Fig. 10), with the appearance of tiny podosomes (Fig. 11A,B,C, arrows). Focal adhesions as well as stress fibers in these cells appeared smaller and thinner as compared with those in normal 3Y1 cells (see Fig. 1A,B). In 6 hours, the peripheral adhesions identified by VNR labeling and the IRM image were observed in 35% of the cells, and this population was nearly the same as for focal adhesions (Fig. 10). In 12 hours, cells forming the peripheral adhesions (Fig. 11E, F, arrowhead) increased (approximately 50%), while the number of cells with focal

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**Fig. 5.** Disruption of the peripheral adhesions of BY1 cells by GRGDSP (200 µg/ml) treatment for 5 minutes. The cells were treated with GRGDSP peptide (the right column), or GRGESSP peptide as a control (the left column). After the treatment, the cells were fixed and subjected to double labeling with the antibody to the VNR (A,B) and rhodamine-phalloidin (C,D). (E and F) The IRM images corresponding to the same cells. In the GRGESSP-treated cells, the peripheral adhesions identified by VNR labeling (A) and the IRM image (E), as well as podosomes detected by F-actin aggregates (C) and the IRM image (E) are preserved intact. In the cells treated with GRGDSP peptide, most of the peripheral adhesions are disrupted (B,F) and only scarce punctuate VNR labeling (B,F, arrows) is left. Note that the drastic change of the actin cytoskeleton is observed in BY1 cells accompanying the destruction of the peripheral adhesions, where podosomes almost disappear (D). Bar, 30 µm.
Adhesive structures in RSV-transformed cells decreased to less than 20% (Fig. 10). In 24 hours, the peripheral adhesions were observed in 70% of cells (Fig. 10). In 36 hours, formation of the peripheral adhesions proceeded along the cell periphery in the majority of the cells (Fig. 11H, I, arrowheads), and such cells showed round or spindle-shaped morphology. The VNR was more enriched.

Fig. 6. Double fluorescence images of BY1 cells stained with the antibody to the VNR (green) and rhodamine-conjugated phalloidin (orange) by confocal laser scan microscopy. Fluorescence images at every 0.5 µm from the cell base (A) to the dorsal side (F) are presented. A series of arrows or arrowheads corresponds to one podosome, respectively. Similarly, a series of large arrows corresponds to one peripheral adhesion. Note the presence of transverse actin bundles (slice E, large arrow) 3-4 slices (about 1.5-2 µm) just above the peripheral adhesions. Bar, 30 µm.
in the peripheral adhesions than in podosomes (compare arrows and arrowheads in Fig. 11E,H). Podosomes became more prominent in size and number with the development of the peripheral adhesions (Fig. 10, compare arrows in Fig. 11A,B,C with D,E,F and G,H,I). On the other hand, it became difficult to identify podosomes by IRM image, as the formation of the peripheral adhesions proceeded (compare arrows in Fig. 11C,F and I). We also cultured BY1 cells for the same period on VN-coated coverslips in the presence of serum, but no remarkable difference was noted as to the kinetics of cell adhesion when compared with the control (coating-free) culture (data not shown).

**Similar peripheral adhesions in cells with poorly organized stress fibers and focal adhesions**

Close contacts at the cell periphery have been observed in motile cells such as leukocytes and epidermal cells (Kolega et al., 1982), and in normal fibroblasts at their dynamic motile stage (Chen, 1981) or the early spreading stage (David-Pfeuty, 1985). In these cells, as well as in RSV-transformed cells, stress fibers and focal adhesions are poorly organized as compared with normal fibroblasts at their stationary phase. We investigated the cell/substratum adhesive structures of such cells, which poorly form stress fibers and focal adhesions. Most 3Y1 cells formed focal adhesions at their stationary phase (as shown in Fig. 1A,B and Fig. 3C,D), however, the peripheral adhesions with the VNR cluster were predominantly formed in some 3Y1 cells at their logarithmic growth phase (Fig. 12A,B, arrowheads). Similar adhesive structures were also observed in phorbol ester-treated rat NRK cells (Fig. 12C,E, arrowheads), and in several malignant tumor cells, such as human epidermal carcinoma KB cells (Fig. 12F,G arrowheads) and in human osteosarcoma MG-63 cells (Fig. 12H,I,J, arrowheads).
**DISCUSSION**

Here we have characterized the peripheral adhesion of RSV-transformed cells and other types of cells, which have poorly organized stress fibers and focal adhesions. The peripheral adhesion apparently differs from the focal adhesion in morphology. IRM images distinguished the peripheral adhesion from the focal adhesion since the former is associated with regions of close contact. The prominent stress fibers terminate at the focal adhesion, while no actin bundles could be detected in the peripheral adhesion at light microscopic level. In addition, the peripheral adhesion is more sensitive to GRGDSP treatment than the focal adhesion. These notions suggest that the peripheral adhesion

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**Fig. 9.** BY1 cells were cultured on VN (A-F)- or FN (G-I)-coated glass for 1 hour (A-C) or 3 hours (D-I) in serum-free culture medium and double-stained with the antibody to the VNR (B,E) and rhodamine-phalloidin (A,D), and with the antibody to the FNR (H) and rhodamine-phalloidin (G), respectively. (C,F and I) The IRM pictures corresponding to the cells in the left side. Podosomes with the VNR cluster are observed at the center of the ventral plasma membrane of BY1 cell cultured for 1 hour on glass coverslips coated with vitronectin (A,B,C, arrows). At this period, a peripheral belt of close contact is also observed, where scarce cluster of the VNR is detected (B,C, arrowheads). In BY1 cells cultured for 3 hours, the VNR cluster are observed in the majority of the peripheral close contacts identified by IRM (E,F, arrowheads). The peripheral adhesions with the FNR cluster are also observed in BY1 cells cultured on fibronectin-coated glass for 3 hours (H,I, arrowheads). Note that the FNR cluster is observed only in some part of the peripheral close contacts. Bars, 30 µm.
might attach to the substratum less stably than the focal adhesion. The peripheral adhesion is also distinct from the podosome. These two adhesive structures are completely different in their morphology. The peripheral adhesion was, as a rule, observed to be darker than the podosome by IRM. Also, the integrin was more enriched in the peripheral adhesion than in the podosome. Podosomes were preferentially located at the center of the ventral plasma membrane, in

**Fig. 10.** The time-course transition in the ratio of the cells bearing podosomes (■), focal adhesions (■), and the peripheral adhesions (●) of BY1 cells in 1, 3, 6, 12, 24 and 36 hours of culture in the presence of 10% fetal calf serum; ordinate, % of cells; abscissa, time (hours). The way of calculating the formation index of each item is described in Materials and Methods.

**Fig. 11.** BY1 cells were cultured for 3 (A,B,C), 12 (D,E,F) and 36 hours (G,H,I) in serum-containing medium and double-stained with the antibody to the VNR (B,E,H) and rhodamine-phalloidin (A,D,G). (C,F and I) are the IRM pictures corresponding to the cells in the left side. Formation of stress fibers and focal adhesions is conspicuous, while podosomes are not prominent in BY1 cells cultured for 3 hours (A,B,C). VNR labeling is detected in focal adhesions (A,B,C, arrowhead) and in podosomes (A,B,C, arrows). In 12 hours, stress fibers and focal adhesions have disappeared and formation of the peripheral adhesions with the VNR cluster are observed (E,F, arrowhead). Podosomes are also prominent at this period (D,E,F, arrows). In 36 hours, the peripheral adhesions are located along the cell periphery (H,I, arrowheads), surrounding the center of the ventral plasma membrane where podosomes are distributed (G,H,I, arrows). Note that the VNR is more enriched in the peripheral adhesions than in podosomes (E,H, arrows and arrowheads). Bar, 30 µm.
Formation of similar adhesive structures to the peripheral adhesions with the VNR cluster in 3Y1 cells at their logarithmic growth phase (A,B), phorbol ester-treated rat NRK cells (C,D,E), human epidermal carcinoma KB cells (F,G) and human osteosarcoma MG-63 cells (H,I,J). 3Y1 cells and KB cells were double-stained with the antibody to the VNR (A,F) and vinculin (B,G). Vinculin labeling was used as a marker of focal adhesions (where stress fibers are terminated). Phorbol ester-treated NRK cells and MG63 cells were double-stained with antibody to VNR (C,H) and rhodamine-phalloidin (D,I). (E and J) are the IRM pictures corresponding to the cells on the left side. In all cells, the VNR cluster was observed in similar adhesive structures to the peripheral adhesions of BY1 cells without associating with any stress fibers (A-J, arrowheads). Bar, 30 μm.
contrast to the peripheral adhesions at the cell periphery. These morphological observations suggest that the peripheral adhesions is a peculiar cell/substratum adhesive structure.

As we have previously reported, contractile proteins such as myosin, caldesmon and tropomyosin are highly concentrated in podosomes, but these proteins were not found in the peripheral adhesions or focal adhesions (Tanaka et al., 1993). The lack of contractile proteins in the peripheral adhesions may be due to their immotility, or a stable adhesiveness similar to focal adhesions. Such findings taken together with the higher IRM image suggest that the peripheral adhesions may play a more important role in the adhesiveness of BY1 cells than podosomes. The peripheral adhesions of BY1 cells may participate in the motility of BY1 cells. Indeed, the active movement of podosomes has been reported (Chen et al., 1989). Besides, it has long been known that podosomes are able to degrade the extracellular matrix by secreting proteases (Chen, 1985; Chen, 1989; Mueller et al., 1992). Therefore, podosomes might be mainly involved in moving proteins, leaving adhesive functions to the peripheral adhesions. As mentioned above, the peripheral adhesions were observed to be less stably adhered to the substratum than focal adhesions of normal fibroblasts. This might be of advantage for actively moving cells such as transformed or malignant cells, and normal cells at their motile or mitotic phase.

In typical cases, the peripheral adhesions form a continuous peripheral belt as if to seal off the center area of the cell, where podosomes are present. A similar adhesion mode is observed in osteoclasts in their bone-resorption phase. They form a belt-like tight adhesion area, so called ‘sealing zone’ at the peripheral margin, and seal off the ‘ruffled border’, within which bone resorption occurs (Raize, 1992). Sealing the substratum subject to degradation would be advantageous in maintaining the local concentration of degradative enzymes at a high level, facilitating the destructive process, in addition to detection of the change in chemical parameters in a degradative environment. Thus, the co-expression of the peripheral adhesions and podosomes might be important for the invasive activity of some malignant cells.

We have examined the expression of the FNR and the VNR in the peripheral adhesions of BY1 cells. The predominant type of integrin expressed in these structures was determined by the substratum. In the presence of serum, the VNR was predominantly expressed in the peripheral adhesions, while the FNR cluster was scarcely detectable. This is in contrast with focal adhesions of normal 3Y1 cells, in some of which the FNR cluster was observed as well as the VNR cluster. The preferential expression of VNR in the peripheral adhesions of BY1 cells may be attributable to the following two reasons; the VNR is predominantly involved in cell/substratum adhesion in the presence of serum (Fath et al., 1989). Furthermore, expression of the FNR is suppressed upon RSV transformation as previously reported (Plantefaber and Hynes, 1989), while that of the VNR is preserved as we demonstrated.

In contrast to focal adhesions of normal fibroblasts, the peripheral adhesions scarcely associated with thick actin bundles. It is known that the cell/substratum adhesion is regulated by the action of protein kinases (Burridge, 1986). In BY1 cells, the peripheral adhesions contain much larger quantities of pp60v-src and tyrosine-phosphorylated proteins than focal adhesions. Tyrosine kinase has been considered to associate intimately with the cytoskeleton and with morphological transformation (Hamaguchi and Hanafusa, 1987). Thus, some regulatory proteins whose functions can be controlled by tyrosine kinases, including pp60v-src, might be responsible for the formation of the peripheral adhesions in BY1 cells. It seems difficult to attribute the development of the peripheral adhesions in the cells other than RSV-transformed cells to one mechanism; elevated phosphorylation has been observed in normal cells at their mitotic phase (Lewin, 1990) and protein kinase C is activated by phorbol ester-treated cells (Castagna et al., 1982). In malignant tumor cells, protein kinases encoded by several oncogenes may also be activated. It may, however, be possible that tyrosine kinases such as the cellular equivalent of the viral protein kinase are similarly involved in the formation of the peripheral adhesions.

It takes much longer (more than 10 hours) for BY1 cells to generate the peripheral adhesions and podosomes in serum-containing medium as opposed to the serum-free culture condition. BY1 cells displayed a two-step mode of adhesion via the integrins; focal adhesions were transiently formed at the early stage of adhesion, afterwards the peripheral adhesions were predominantly observed. Such kinetics of adhesion of BY1 cells were not affected by the presence of precoated adhesion molecules. Thus, we speculated that some growth factors or adhesion molecules in serum might promote the formation of stress fibers together with focal adhesions, and delay the formation of the peripheral adhesions and podosomes at the early stage of adhesion. We cultured BY1 cells in the presence of serum with several changes of medium at short intervals for minimizing environmental change such as pH change, or consumption of some growth factors in the culture medium. However, little difference was observed in the formation of the peripheral adhesions when compared with the control experiments (data not shown). This suggests that the peripheral adhesions of BY1 cells may not be such transient forms of adhesive structures as a consequence of the environmental change in culture conditions, but are intrinsic and stable adhesive structures.

Future directions are to investigate the regulatory mechanisms of the peripheral adhesions, especially their functional interaction with podosomes in cell adhesion and invasion of BY1 cells, which would give further insights into the mysterious behavior of malignant tumor cells in the metastatic cascade, in that they possess incompatible characteristics, are highly adherent, and at the same time invasive to the host tissue that is preferentially subject to metastasis (Nicolson, 1988).

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