An active form of sphingosine kinase-1 is released in the extracellular medium as component of membrane vesicles shed by two human tumoral cell lines.

Salvatrice Rigogliuso*, Chiara Donati°, Donata Cassarà*, Simona Taverna*, Monica Salamone#*, Paola Bruni° and Maria Letizia Vittorelli*°

* From the Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Palermo, Viale delle Scienze ed. 16, 90128 Palermo, Italy.

° From the Dipartimento di Scienze Biochimiche, Università di Firenze, Viale G.B. Morgagni n. 50, 50134 Firenze, Italy.

• Present address: Dip. Biopatologia e Metodologie Biomediche Università di Palermo, Via Divisi 83, 90133 Palermo, Italy.

# Present address: IAMC-CNR, U.O. Capo Granitola, Mazara del Vallo, 91026 Trapani, Italy.

• To whom correspondence should be addressed. Tel 39-0916811125. E-mail mlvitt@unipa.it
Abstract

Expression of sphingosine kinase-1 (SphK-1) correlates with poor survival of tumor patients. This effect is probably due to the capability of SphK-1 to be released into the extracellular medium where it catalyzes the biosynthesis of sphingosine 1-phosphate (S1P), a signalling molecule endowed with profound proangiogenic effects. SphK-1 is a leader-less protein and it is secreted by unconventional mechanism. We report that in human hepatocarcinoma Sk-Hep1 cells, targeting of the enzyme to the cell surface is induced by extracellular signalling and parallels targeting of FGF-2 to the budding vesicles. We also show that SphK-1 is present in a catalytically active form in vesicles shed by SK-Hep1 and human breast carcinoma 8701-BC cells. The enzyme substrate sphingosine is present in shed vesicles where it is produced by neutral ceramidase. Shed vesicles are therefore a site for S1P production in the extracellular medium, and conceivably also within host cell, after vesicle endocytosis.
Introduction.

Malignant tumors have the remarkable ability to modulate their stromal environment to their own advantage. They alter surrounding extracellular matrix and modify normal cell behaviour to facilitate tumor cell growth, invasion, immune evasion and angiogenesis [1]. Most of these effects are mediated by the release of small vesicles from the tumor cells into the extracellular medium. Shed vesicles are known to facilitate tumor invasion [2-4], mainly acting by proteolytic enzymes associated with their membrane [5-9]. Indeed, the vesicle membranes are selectively enriched in some components including MMP-9 [7] and other proteolytic enzymes [4, 6], together with β1 Integrin and class I HLA molecules [7]. Enrichment of ganglioside GD3 and caveolin-1 was also reported [10]. Moreover vesicles contribute, by several mechanisms, to tumor escape from immune reactions [11-16].

Notably, vesicles carry many proangiogenic growth factors, differently expressed depending on the vesicle origin, that act on endothelial cells thus promoting angiogenesis. Indeed, FGF-2 was detected in vesicles shed by human hepatocarcinoma Sk-Hep1 cells [17, 18]; VEGF was found to be present in vesicles shed by human ovarian carcinoma cells [19] and in vesicles shed by neurons and astrocytes [20, 21]; angiogenin, IL-6, IL-8, VEGF and TIMPs were found in vesicles shed by glioblastoma tumor cells [22]. Additionally, the sphingolipid fraction of vesicles shed by HT1080 fibrosarcoma and DU-145 human prostate carcinoma cells was also shown to exert proangiogenic activity [23].

Sphingomyelin is a normal component of plasma membranes where is normally clustered in the outer membrane leaflet. It is subjected to intense metabolism responsible for the formation of a number of bioactive metabolites including ceramide, ceramide-1-phosphate, sphingosine and sphingosine 1-phosphate (S1P) [24]. Ceramide, generated by sphingomyelinase (SMase) action on sphingomyelin, appears to be a critical regulator of cell growth arrest, differentiation and apoptosis [25, 26]. Sphingosine is formed by ceramide deacylation catalyzed by at least three different isoforms of ceramidase, which differ for optimal pH, primary structure and cellular localization.
The enzyme sphingosine kinase (SphK) catalyzes the formation of S1P from sphingosine and ATP [28]. Two distinct SphK isoforms, SphK-1 and SphK-2, have been cloned [29, 30]. SphK-1, the more deeply investigated isoform, is primarily localized in the cytosol, but, following ERK-dependent phosphorylation elicited by various stimuli, it becomes associated to plasma membrane [31]. SphK-1 has been shown to regulate a wide variety of cellular processes, including promotion of cell proliferation, survival and motility [32] and, importantly, possesses oncogenic potential [33]. Previous studies have established that SphK-1, similarly to FGF-2 and several other proteins, can be released in the extracellular environment although lacking a conventional secretory signal sequence. The mechanism of SphK-1 secretion is unconventional and likely involves a non classical pathway independent of the endoplasmic reticulum/Golgi system; SphK-1 secretion mechanism is only known to require functional actin dynamics [34]. Notably, the SphK product S1P, among the multiple biological activities, exerts a strong proangiogenic action which is known to act synergistically with growth factors such as FGF-2 [35, 36]; and VEGF [35].

In this study we investigated whether vesicles shed by hepatocarcinoma and carcinoma cultured cells contain S1P-generating enzymes. Presented data demonstrate that neutral ceramidase (nCDase) as well as SphK-1 are localized in vesicles supporting the view that S1P participates to the proangiogenic activity exerted by these particles.
Materials and Methods

**Cells and Culture Media.** Human SK-Hep1 hepatocarcinoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS; Euroclone, Celbio). Human breast carcinoma 8701-BC cells, kindly provided by Prof S. Minafra [37] were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Euroclone, Celbio). Bovine GM7373 fetal aortic endothelial cells were grown in Eagle’s minimal essential medium (Euroclone, Celbio) supplemented with 10% FCS, vitamins, and essential and non-essential amino acids.

**Cell extraction.** Cells were removed from plate by a scraper and centrifuged at 2000g for 5 min; pelleted cells were then resuspended in 300µl of Triton X100 (1%) on phosphate buffer saline (PBS). After 10 min incubation at room temperature, cell extract was centrifuged at 800g for 10 min. The amount of protein extracted from cells was determined using the Bradford microassay method (Bio-Rad, Segrate, Milan, Italy) employing bovine serum albumin (Sigma-Aldrich) as a standard.

**Vesicle Purification from Conditioned Medium.** Vesicles were purified from conditioned medium as previously described [38]. Briefly, medium conditioned by culturing sub confluent healthy cells for 3 or 24 h, were centrifuged at 2000g for 10 min and at 4000g for 15 min. The supernatant was ultracentrifuged at 105,000g in Ti-60 Rotor (Backman) for 90 min. Pelleted vesicles were resuspended in PBS. The amount of isolated vesicles was determined measuring the protein concentration through the Bradford microassay method (Bio-Rad, Segrate, Milan, Italy) using bovine serum albumin (Sigma-Aldrich) as a standard.

**Western Blotting.** After SDS-PAGE electrophoresis in 7.5% gels, the proteins were blotted onto a nitrocellulose membrane (Hybond; Amersham Biosciences) that was saturated with 3% non fat dry milk on Tris Buffer Saline 50mM pH 7.9/Tween 0,05% (TBS-T). After 5 washes in TBS-T for 5 min each, the nitrocellulose membranes were incubated overnight at 4°C, with mouse monoclonal anti-nCDase antibody 1:200; (a kind gift of Prof. M. Ito, Fukuoka, Japan) [39]. The primary antibody was followed by peroxidase-conjugated anti-mouse antibodies (1:10000) (Amersham Biosciences) for 1h at room temperature. Immunocomplexes were visualized with the ECL Western blotting kit (Amersham Biosciences) using Hyperfilms.
Confocal Immunofluorescence. Cells, seeded at low density (2,000 cells/well) onto microscope cover slips in 12-well culture plates (Nunc), were grown overnight in complete medium and, when needed, for 3 more days in serum-free medium with three medium changes. SphK-1 and SphK-2 were detected using as primary antibodies respectively rabbit polyclonal anti-SphK-1 antibody (a kind gift of Prof. L. Obeid, Charleston, SC, USA) [40] 1:100 and rabbit polyclonal anti-SphK-2 antibodies 1:100 (kindly provided by Dr. S. Nakamura, Kobe, Japan) [41] and as secondary antibodies anti rabbit TRITC conjugated antibodies (1:200 Sigma); β1 Integrin was detected using C27 anti β1 Integrin rat primary monoclonal antibody 1:150, [42] and anti rat TRITC conjugated secondary antibody (1:320, Sigma). FGF-2 was detected using mouse monoclonal anti FGF-2 antibody (0.5 mg/ml 1:200, Upstate Biotechnology type II) and Texas Red-conjugated anti-mouse antibody (1:200, Amersham Biosciences).

In order to stain nuclei, cells were fixed in 3.7% formaldehyde and than stained for 10 min with propidium iodide (Sigma).

Immunostained cells were analyzed by confocal microscopy (Olympus 1X70 with Melles Griot laser system).

Staining of vesicle lipids. Vesicle lipids were stained with the lipophilic styryl compound FM4-64 (Molecular Probes). Purified vesicles (180 µg) were resuspended in 1ml PBS and stained with FM4-64 dissolved in PBS without calcium and magnesium. FM4-64 was added at a final concentration of 5 µg/ml; samples were incubated at room temperature for 15 min. Stained vesicles were collected by centrifugation at 50,000g for 1h, resuspended in 50µl PBS and added to GM7373 cells to monitor vesicle targeting.

Transient cell transfection. SK-Hep1 cells were plated in six-well culture plates at 3x10^5 cells/well and maintained overnight in high-glucose DMEM containing 10% fetal calf serum, 2mM L-glutamine, 100 IU/ml penicillin and 100µg/ml streptomycin. The next day, cells were transfected with SKpeGFP plasmid encoding for SphK-GFP chimera (a kind gift of Prof. S. Spiegel, Richmond, VA, USA) [43]. Transfection was carried out using Lipofectamine Reagent (GIBCO Life Technologies), according to the manufacturer’s instructions.

Sphingosine kinase assay. SphK activity was assayed in isolated vesicles or serum free conditioned medium as described by Olivera et al. [28]. Briefly, 50 µg vesicle proteins were resuspended in 100 µl of the reaction mixture which contained 20mM Tris–HCl, pH 7.4, 20% (v/v) glycerol, 1mM β-mercaptoethanol, 1mM EDTA, 1mM sodium orthovanadate, 15mM sodium fluoride, protease
inhibitors (10 mg/ml leupeptin, 10 mg/ml aprotinin, and 1mM PMSF) and 0.5 mM 4-deoxypyridoxine.

Serum free conditioned medium was concentrated approximately 40-fold before to be employed for enzymatic activity measurement.

The enzymatic reaction was initiated by adding 50 μM sphingosine and 1 mM [γ32P] ATP. In some cases assays were performed omitting sphingosine, to evaluate availability of endogenous sphingosine. After 30 min incubation at 37° C., the reaction was terminated by addition of 20 μl 1 N HCl and 900 μl of chloroform/methanol/HCl (100:200:1, v/v). Lipids were then extracted, separated by TLC and labelled S1P quantified by liquid scintillation essentially as previously described [44]. Specific activity of SphK was expressed as pmol of S1P produced/min/mg of protein.

Neutral ceramidase activity assay

nCDase activity was determined using C12-NBD-ceramide as a substrate as previously described [45]. Briefly, 100 pmol of C12-NBD-ceramide (NBD-C12:0, d18:1) were incubated 2 h at 37 °C with an appropriate amount of proteins in 20 μl of 25mM Tris-HCl buffer pH 7.5 and 0.25% (w/v) Triton X-100. Samples were then applied to a TLC plate, which was developed with chloroform, methanol, 25% ammonia (90:20:0.5, v/v). Spots corresponding to NBD-dodecanoic acid and C12-NBD-ceramide were scraped, incubated with methanol at 37 °C to extract the compounds from the silica and their fluorescence at 470/525 nm excitation/emission wavelengths was measured using a Shimadzu 9000 spectrophotofluorimeter. The compounds were quantified using a standard curve of known amounts of C12-NBD-ceramide and NBD-dodecanoic acid.
Results.

Immunolocalization of SphK-1 and Sphk-2 in 8701-BC and Sk-Hep-1 cells.

In a first group of experiments expression and localization of SphK-1 and SphK-2 were analyzed by immunofluorescence in 8701 BC carcinoma cells and in Sk-Hep1 hepatocarcinoma cells (Figure 1).

A) localization in 8701-BC cells
B) localization in Sk-Hep1 cells

**Line a:** Immunolocalization of β1 Integrin and SphK-2 showing a different distribution of the two molecules.

**Line b:** Immunolocalization of β1 Integrin and SphK-1. Arrows indicate co-localization areas.

β1 Integrin was detected using FITC-conjugated secondary antibodies, SphK-2 and SphK-1 using Texas red-conjugated secondary antibodies. Arrows indicate co-localization areas.

Figure 1: Compared analysis of β1 Integrin, SphK-2 and SphK-1 immunolocalization.
Moreover, since it had been previously demonstrated that β₁ integrin is clustered in shed vesicles [7, 17], distribution of the two proteins was compared with distribution of β₁ integrin.

In both cell lines distribution of SphK-2 was quite different from distribution of β₁ integrin (Figure 1A and 1B line a), SphK-2 indeed was clustered in the cell nucleus whereas it was absent at the cell membrane where instead β₁ integrin was located. In contrast, SphK-1 and β₁ integrin appear to co-localize at the plasma membrane (Fig.1 A and 1B lines b). Moreover, as it can be more clearly observed in cells transiently transfected with GFP-SphK-1 (Figure 2), both SphK-1 and β₁ integrin seem to be more densely packed in specific areas of the plasma membrane, and clustering appears to occur in areas of the cell membrane from which vesicles are released (Fig.2 panel d).

**Figure 2: Compared analysis of SphK-1 and of β₁ Integrin localization in transfected Sk-Hep1 cells.**

- **a):** GFP-bound SphK-1 localization in transfected cells. The protein is localized in cell membranes where it shows an uneven clustering in small spots.
- **b):** Immunolocalization of β₁ Integrin detected using Texas red-conjugated secondary antibodies. β₁ Integrin is seen in cell membranes of both transfected and non transfected cells. Like SphK-1, β₁ Integrin shows an uneven clustering in small spots.
- **c):** Double staining shows co-localization of the two proteins in some areas of the plasma membrane. (Indicated by arrows.)
- **d):** Enlargement of a cell protrusion showing budding areas (indicated by arrows) in which SphK-1 appears to have a preferential localization.

**Effects of serum addition on SphK-1 trafficking toward the cell periphery.**

In a previous work we observed that vesicles shed by Sk-Hep1 cells mediate FGF-2 release and that vesicle shedding and release of FGF-2 were simultaneously induced by addition of serum to starved cells [17]. By monitoring intracellular movements of the growth factor subsequent to serum addition, we showed that FGF-2 within one hour was targeted to the cell periphery and to the cell nucleus and nucleolus. FGF-2 movements toward the cell periphery required actin filament integrity [18]
Similarly to FGF-2, SphK-1 is a leader-less protein secreted by unconventional mechanisms [29, 34] whose export toward the cell periphery is mediated by actin filaments [34]. We therefore considered that the two proteins could share a similar export mechanism and analyzed whether intracellular movements of SphK-1 were influenced by serum addition and whether the enzyme co-localized with FGF-2.

Intracellular distribution of SphK-1 was therefore analyzed by immunolocalization in starved cells as well as at time intervals after serum addition and compared with FGF-2 distribution. As shown in Figure 3, in starved cells the two proteins did not co-localize, being SphK-1 partially localized in small granules and FGF-2 totally dispersed.

**Figure 3.** Time-course of endogenous SphK-1 and endogenous FGF-2 targeting to the cell periphery, observed by immunolocalization experiments.

FGF-2 and SphK-1 immunolocalization at 0, 30 and 60 min after serum addition (lines a, b, c respectively). Sections at 3 μm from surface.

FGF-2 was detected using Texas red conjugated secondary antibodies; SphK-1 was detected using FITC-conjugated antibodies. Arrows indicate granules of protein localization.
Thirty minutes after serum addition both proteins were clustered in granules and showed a clear co-localization. After 1 h from serum addition large granules positive for both proteins were present near to the cell membrane. In contrast, cell nuclei were exclusively stained by anti FGF-2 antibodies.

These results suggest that FGF-2 and SphK-1 share a similar transport mechanism toward the cell periphery and that the two proteins are likely to be both targeted to the budding vesicles.

Detection of an active form of SphK-1 in shed vesicles.

In order to establish if an active form of SphK-1 is shed as a component of membrane vesicles, in some experiments we determined SphK activity in vesicles or cell conditioned medium. Results reported in Table 1 show that SphK-1 was clearly detectable in vesicle shed by Sk-Hep1 and 8701-BC cells, although the specific activity was found to be higher in vesicles shed by Sk-Hep1 cells.

Table 1

| Sample            | Incubation mixture with exogenous sphingosine | Incubation mixture without exogenous sphingosine |
|-------------------|-----------------------------------------------|-----------------------------------------------|
| SK-Hep1 vesicles  | 43.93 pmol/min/mg of protein                   | 26.70 pmol/min/mg of protein                   |
| SK-Hep1 vesicles* | 43.59 pmol/min/mg of protein                   |                                               |
| SK-Hep1 C.M.      | 2.23 pmol/min/mg of protein                    |                                               |
| 8701-BC vesicles  | 14.44 pmol/min/mg of protein                   | 12.61 pmol/min/mg of protein                   |
| 8701-BC vesicles* | 16.70 pmol/min/mg of protein                   |                                               |
| 8701-BC CM        | 2.16 pmol/min/mg of protein                    |                                               |

* Vesicles collected from medium in which 2M NaCl had been added.

SphK-1 activity was also tested in vesicle-deprived conditioned medium. No enzymatic activity could be detected in serum-containing medium, even when it was concentrated. Instead, it was possible to detect a low SphK activity in 40-fold concentrated serum-free medium conditioned by maintaining cells in culture for 24 h. As shown in Table 1 when vesicles were collected from medium to which 2M NaCl had been added, in order to solubilize proteoglycans and other molecules unspecifically bound to the vesicle membrane, the enzymatic activity was not affected.
Addition of sphingosine increased labeled S1P production, however the catalytic activity of the enzyme was observed also when this substrate was not added. This result indicates that sphingosine is already present in shed vesicles as a membrane component.

Sphingosine is likely produced by nCDase, which is known to be localized in plasma membranes and also found in the extracellular medium [46, 47]. nCDase was actually found to be present in an enzymatically active form in vesicles shed by 8701 BC, (Figure 4 and Table 2). Moreover, in Sk-Hep1 cells we observed co-localization of nCDase and β1 integrin (data not shown), indicating that nCDase is likely to be present also in vesicles shed by this cell line.

![Western Blot analysis for nCDase (A) and its densitometric analysis (B) on 8701BC vesicles and cell extracts.](image)

### Table 2

| Sample            | Enzymatic activity of nCDase          |
|-------------------|---------------------------------------|
| 8701-BC cells     | 2.08 pmol/min/mg of protein           |
| 8701-BC vesicles  | 5.25 pmol/min/mg of protein           |
Fate of shed vesicles.

Since S1P can be produced at the membrane of shed vesicles the molecule could remain, at least in part, within the vesicle. Vesicles could adhere to the plasma membrane of cells surrounding the tumor and S1P could exert its effects by interacting with receptors localized at the cell surface. On the other hand, vesicles could be internalized by the host cell and consequently release S1P inside cells where it could act as intracellular messenger. In order to verify these hypotheses we analyzed targeting of shed vesicles after adding them to GM7373 cells, an immortalized line of embryonic bovine aortic endothelial cells.

For this purpose vesicles released by Sk-Hep1 cells were labelled for 15 min with the lipid marker FM4-64. Labelled vesicles were then added to in vitro cultured GM7373 cells which on turn had been labelled with antibodies against β1 integrin. As shown in Figure 5, at 10 min incubation vesicles were observed to be bound to the cell membrane, while at 20 min incubation most vesicles were internalized and visible in the cytoplasm. At 30 min the signal borne by lipid marker FM4-64 was no more visible indicating that the lipids of the vesicle membranes were degraded.

In principle therefore, vesicle-associated S1P could act both on membrane receptors and intracellular targets.

Figure 5. Interactions of shed vesicles with endothelial cells.
Vesicles shed by SK-Hep1 cells, labelled with lipid styryl dye FM4-64 (Red fluorescence), were added to GM7373 endothelial cells in which β1 Integrin was stained using FITC-conjugated secondary antibodies (Green fluorescence). Cells were incubated with vesicles respectively for 10 min. (a); 20 min. (b); 30 min (c). The arrows indicate vesicle localization.
Conclusions

Membrane vesicles shed by tumor cells appear to exert a variety of effects on the surrounding cells. Vesicles are rich of enzymatic activities able to modify the extracellular medium composition that’s facilitating tumor cell migration and angiogenesis. Depending on their origin, they also convey different signalling molecules which exert their effects on lymphocytes, mesenchymal cells and endothelial cells. Shed vesicles have been shown to induce angiogenesis by a variety of mechanisms including the action of proteins such as FGF-2, VEGF, angiogenin, IL-6, IL-8 and TIMPs and molecules of lipid nature such as sphingomyelin.

Based on the present results nCDase and SphK-1 can now be included among the signalling molecules transferred by shed membrane vesicles suggesting that S1P formed at level of the vesicle membranes plays a role in the biological processes regulated by these particles. Interestingly, nCDase, here recognized as component of shed vesicles, was previously identified in various subcellular compartments such as endosomes, mitochondria and microdomains of the plasma membrane [48, 39], but also found involved in extracellular sphingolipid metabolism [47]. In this regard it was demonstrated that although nCDase is localized at plasma membrane as a type II integral membrane protein, the enzyme is released in the extracellular medium after the proteolytic action of secretases [49, 50]. Moreover, in agreement with the present results, nCDase, together with acid SMase, was identified as component of a complex at cell membrane domain subjected to budding as well as in conditioned medium associated with caveolin-1, a key structural protein of caveole [51] which was also detected in shed vesicles [52].

SphK-1 is a secreted leader-less protein and shedding of membrane vesicles appears to represent a mechanism which accounts for its secretion. The presence of the enzymatic protein in shed vesicles does not per se exclude that other mechanisms can also participate to the release of SphK-1. Indeed it was reported that, in FGF-1 overexpressing NIH 3T3 cells, SphK-1 is secreted together with FGF-1, as component of a high molecular weight complex [53, 54]. SphK-1 however is secreted also by cells which do not express FGF-1 and also in absence of stress signalling which induces FGF-1 secretion. Here we show that, at least in some instances, SphK-1 is secreted as a component of shed vesicles. Since shed vesicles also contain nCDase which, by catalyzing sphingosine generation, provides the rate-limiting substrate for S1P production, it is likely that these particles bring about sustained S1P production.

SphK-1 and S1P produced by its enzymatic activity are able to mediate a network of paracrine signalling. It is well-known that acting on the two membrane receptors S1P₁ and S1P₃, S1P induces morphogenesis in HUVEC cells [35]. Moreover, since vesicles carry several other molecules able to
affect angiogenesis, the overall effects of vesicles on surrounding endothelial cells will be amplified and differently modulated depending on the specific composition of vesicles.

The exact mechanism by which the S1P message borne by shed vesicles is delivered to the host cell remains to be explored. Indeed, an attractive hypothesis is that after interacting with the recipient cell plasma membrane, vesicles are internalized via endocytosis. SphK-1 and SIP would therefore be delivered into the cytoplasm of the receiving cell, where, as already known, SIP could exert its intracellular effects, regulating various processes among which cell survival is prominent [55, 56]. Indeed vesicles were shown to convey molecules, such as mRNA and iRNA, to the cytoplasm of surrounding cells, and it was recently reported that mRNA included in shed vesicles can be translated in recipient cells following endocytosis [22].

Alternatively, since SphK-1 association with cell membrane following its phosphorylation is strengthened by interaction with phosphatidylinerine [57] and exposure of phosphatidylinerine on the outer leaflet is a hallmark of shed membrane vesicles [58, 59], it can also be speculated that SphK-1, localized in these particles, can generate SIP in the extracellular environment. If this is the case, the bioactive lipid generated outside the endothelial cells could determine key effects on development and proliferation of endothelial cells, acting as ligand of S1P_1 and/or S1P_3 receptors [60, 61]. Independently from the mechanism of action, the here discovered feature of shed vesicles to carry on key enzymes for SIP production enlightens a novel aspect of their biochemical properties which can be relevant for the complete understanding of their pro-angiogenic activity.

**Acknowledgments**

This work was supported by the Italian Ministry of University and Scientific and Technological Research (to M.L.V. and P.B.) and by grants from University of Florence and “Ente Cassa di Risparmio di Firenze” (to P.B.).

We would like to thank Salvatore Agnello for his technical assistance with the confocal microscopy. We also thank Prof. Salvatore Minafra for providing us with 8701-BC cells.
References

1. Singer, C. F., Gschwantler-Kaulich, D., Fink-Retter, A., Haas, C., Hudelist, G., Czerwenka, K., and Kubista, E. Differential gene expression profile in breast cancer-derived stromal fibroblasts. Breast Cancer Res.Treat., 110: 273-281, 2008.

2. Poste, G. and Nicolson, G. L. Arrest and metastasis of blood-borne tumor cells are modified by fusion of plasma membrane vesicles from highly metastatic cells. Proc.Natl.Acad.Sci.U.S.A, 77: 399-403, 1980.

3. Taylor, D. D., Taylor, C. G., Jiang, C. G., and Black, P. H. Characterization of plasma membrane shedding from murine melanoma cells. Int.J.Cancer, 41: 629-635, 1988.

4. Ginestra, A., La Placa, M. D., Saladino, F., Cassara, D., Nagase, H., and Vittorelli, M. L. The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. Anticancer Res., 18: 3433-3437, 1998.

5. Zucker, S., Wieman, J. M., Lysik, R. M., Wilkie, D. P., Ramamurthy, N., and Lane, B. Metastatic mouse melanoma cells release collagen-gelatin degrading metalloproteinases as components of shed membrane vesicles. Biochim.Biophys.Acta, 924: 225-237, 1987.

6. Ginestra, A., Monea, S., Seghezzi, G., Dolo, V., Nagase, H., Mignatti, P., and Vittorelli, M. L. Urokinase plasminogen activator and gelatinases are associated with membrane vesicles shed by human HT1080 fibrosarcoma cells. J.Biol.Chem., 272: 17216-17222, 1997.

7. Dolo, V., Ginestra, A., Cassara, D., Violini, S., Lucania, G., Torrisi, M. R., Nagase, H., Canevi, S., Pavan, A., and Vittorelli, M. L. Selective localization of matrix metalloproteinase 9, beta1 integrins, and human lymphocyte antigen class I molecules on membrane vesicles shed by 8701-BC breast carcinoma cells. Cancer Res., 58: 4468-4474, 1998.

8. Taraboletti, G., D'Ascenzo, S., Borsotti, P., Giavazzi, R., Pavan, A., and Dolo, V. Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. Am.J.Pathol., 160: 673-680, 2002.

9. Graves, L. E., Ariztia, E. V., Navari, J. R., Matzel, H. J., Stack, M. S., and Fishman, D. A. Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. Cancer Res., 64: 7045-7049, 2004.

10. Dolo, V., Li, R., Dillinger, M., Flati, S., Manela, J., Taylor, B. J., Pavan, A., and Ladisch, S. Enrichment and localization of ganglioside G(D3) and caveolin-1 in shed tumor cell membrane vesicles. Biochim.Biophys.Acta, 1486: 265-274, 2000.

11. Alexander, P. Proceedings: Escape from immune destruction by the host through shedding of surface antigens: is this a characteristic shared by malignant and embryonic cells? Cancer Res., 34: 2077-2082, 1974.

12. Poutsiaka, D. D., Schroder, E. W., Taylor, D. D., Levy, E. M., and Black, P. H. Membrane vesicles shed by murine melanoma cells selectively inhibit the expression of Ia antigen by macrophages. J.Immunol., 134: 138-144, 1985.
13. Dolo, V., Adobati, E., Canevari, S., Picone, M. A., and Vittorelli, M. L. Membrane vesicles shed into the extracellular medium by human breast carcinoma cells carry tumor-associated surface antigens. Clin.Exp.Metastasis, 13: 277-286, 1995.

14. Zuccato, E., Blott, E. J., Holt, O., Sigismund, S., Shaw, M., Bossi, G., and Griffiths, G. M. Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation. J.Cell Sci., 120: 191-199, 2007.

15. Clayton, A., Mitchell, J. P., Court, J., Mason, M. D., and Tabi, Z. Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. Cancer Res., 67: 7458-7466, 2007.

16. Gabrilovich, D. I. Molecular mechanisms and therapeutic reversal of immune suppression in cancer. Curr.Cancer Drug Targets., 7: 1, 2007.

17. Taverna, S., Ghersi, G., Ginestra, A., Rigogliuso, S., Pecorella, S., Alaimo, G., Saladino, F., Dolo, V., Dell'Era, P., Pavan, A., Pizzolanti, G., Mignatti, P., Presta, M., and Vittorelli, M. L. Shedding of membrane vesicles mediates fibroblast growth factor-2 release from cells. J.Biol.Chem., 278: 51911-51919, 2003.

18. Taverna, S., Rigogliuso, S., Salamone, M., and Vittorelli, M. L. Intracellular trafficking of endogenous fibroblast growth factor-2. FEBS J., 275: 1579-1592, 2008.

19. Taraboletti, G., D'Ascenzo, S., Giusti, I., Marchetti, D., Borsotti, P., Millimaggi, D., Giavazzi, R., Pavan, A., and Dolo, V. Bioavailability of VEGF in tumor-shed vesicles depends on vesicle burst induced by acidic pH. Neoplasia., 8: 96-103, 2006.

20. Schiera, G., Proia, P., Alberti, C., Mineo, M., Savettieri, G., and Di, L., I Neurons produce FGF2 and VEGF and secrete them at least in part by shedding extracellular vesicles. J.Cell Mol.Med., 11: 1384-1394, 2007.

21. Proia, P., Schiera, G., Mineo, M., Ingrassia, A. M., Santoro, G., Savettieri, G., and Di, L., I Astrocytes shed extracellular vesicles that contain fibroblast growth factor-2 and vascular endothelial growth factor. Int.J.Mol.Med., 21: 63-67, 2008.

22. Skog, J., Wurdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., Curry, W. T., Jr., Carter, B. S., Krichevsky, A. M., and Breakfield, X. O. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat.Cell Biol., 10: 1470-1476, 2008.

23. Kim, C. W., Lee, H. M., Lee, T. H., Kang, C., Kleinman, H. K., and Gho, Y. S. Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. Cancer Res., 62: 6312-6317, 2002.

24. Pyne, S. and Pyne, N. J. Sphingosine 1-phosphate signalling in mammalian cells. Biochem.J., 349: 385-402, 2000.

25. Hannun, Y. A. Functions of ceramide in coordinating cellular responses to stress. Science, 274: 1855-1859, 1996.

26. Kolesnick, R. N. and Kronke, M. Regulation of ceramide production and apoptosis. Annu.Rev.Physiol, 60: 643-665, 1998.
27. Ito, M., Okino, N., Tani, M., Mitsutake, S., and Mori, K. [Molecular evolution of neutral ceramidase: signalling molecule and virulence factor]. Tanpakushitsu Kakusan Koso, 47: 455-462, 2002.

28. Olivera, A., Kohama, T., Tu, Z., Milstien, S., and Spiegel, S. Purification and characterization of rat kidney sphingosine kinase. J.Biol.Chem., 273: 12576-12583, 1998.

29. Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. Molecular cloning and functional characterization of murine sphingosine kinase. J.Biol.Chem., 273: 23722-23728, 1998.

30. Liu, H., Sugiura, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Milstien, S., Kohama, T., and Spiegel, S. Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. J.Biol.Chem., 275: 19513-19520, 2000.

31. Pitson, S. M., Moretti, P. A., Zebol, J. R., Lynn, H. E., Xia, P., Vadas, M. A., and Wattenberg, B. W. Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. EMBO J., 22: 5491-5500, 2003.

32. Spiegel, S. and Milstien, S. Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. Biochem.Soc.Trans., 31: 1216-1219, 2003.

33. Pitson, S. M., Xia, P., Leclercq, T. M., Moretti, P. A., Zebol, J. R., Lynn, H. E., Wattenberg, B. W., and Vadas, M. A. Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling. J.Exp.Med., 201: 49-54, 2005.

34. Ancellin, N., Colmont, C., Su, J., Li, Q., Mittereder, N., Chae, S. S., Stefansson, S., Liau, G., and Hla, T. Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. J.Biol.Chem., 277: 6667-6675, 2002.

35. Lee, M. J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M., Sha'afi, R. I., and Hla, T. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. Cell, 99: 301-312, 1999.

36. Harvey, K., Siddiqui, R. A., Sliva, D., Garcia, J. G., and English, D. Serum factors involved in human microvascular endothelial cell morphogenesis. J.Lab Clin.Med., 140: 188-198, 2002.

37. Minafra, S., Morello, V., Glorioso, F., La Fiura, A., Tomasino, R. M., Feo, S., Mcintos, D., and Wolley, D. E. A new cell-line (8701-BC) from primary ductal infiltrating carcinoma of human breast. Br. J. Cancer, 60: 185-192, 1989.

38. Dolo, V., Ginestra, A., Ghersi, G., Nagase, H., and Vittorelli, M. L. Human breast carcinoma cells cultured in the presence of serum shed membrane vesicles rich in gelatinolytic activities. J.Submicrosc.Cytol.Pathol., 26: 173-180, 1994.

39. Mitsutake, S., Tani, M., Okino, N., Mori, K., Ichinose, S., Omori, A., Iida, H., Nakamura, T., and Ito, M. Purification, characterization, molecular cloning, and subcellular distribution of neutral ceramidase of rat kidney. J.Biol.Chem., 276: 26249-26259, 2001.
40. Johnson, K. R., Becker, K. P., Facchinetti, M. M., Hannun, Y. A., and Obeid, L. M. PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane. Extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (PMA). J.Biol.Chem., 277: 35257-35262, 2002.

41. Igarashi, N., Okada, T., Hayashi, S., Fujita, T., Jahangeer, S., and Nakamura, S. Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis. J.Biol.Chem., 278: 46832-46839, 2003.

42. Monsky, W. L., Lin, C.-Y., Aoyama, A., Kelly, T., Mueller, S. C., Akiyama, S. K., and Chen, W.-T. A potential marker protease of invasiveness, seprase, is localized on invadopodia of human malignant melanoma cells. Cancer Res., 54: 5702-5710, 1994.

43. Olivera, A., Rosenfeldt, H. M., Bektas, M., Wang, F., Ishii, I., Chun, J., Milstien, S., and Spiegel, S. Sphingosine kinase type 1 induces G12/13-mediated stress fiber formation, yet promotes growth and survival independent of G protein-coupled receptors. J.Biol.Chem., 278: 46452-46460, 2003.

44. Donati, C., Cencetti, F., De Palma, C., Rapizzi, E., Brunelli, S., Cossu, G., Clementi, E., and Bruni, P. TGFBeta protects mesoangioblasts from apoptosis via sphingosine kinase-1 regulation. Cell Signal., 21: 228-236, 2009.

45. Romiti, E., Meacci, E., Tani, M., Nuti, F., Farnararo, M., Ito, M., and Bruni, P. Neutral/alkaline and acid ceramidase activities are actively released by murine endothelial cells. Biochem.Biophys.Res.Commun., 275: 746-751, 2000.

46. Hwang, Y. H., Tani, M., Nakagawa, T., Okino, N., and Ito, M. Subcellular localization of human neutral ceramidase expressed in HEK293 cells. Biochem.Biophys.Res.Commun., 331: 37-42, 2005.

47. Tani, M., Ito, M., and Igarashi, Y. Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. Cell Signal., 19: 229-237, 2007.

48. Tani, M., Okino, N., Mori, K., Tanigawa, T., Izu, H., and Ito, M. Molecular cloning of the full-length cDNA encoding mouse neutral ceramidase. A novel but highly conserved gene family of neutral/alkaline ceramidases. J.Biol.Chem., 275: 11229-11234, 2000.

49. Tani, M., Iida, H., and Ito, M. O-glycosylation of mucin-like domain retains the neutral ceramidase on the plasma membranes as a type II integral membrane protein. J.Biol.Chem., 278: 10523-10530, 2003.

50. Yoshimura, Y., Tani, M., Okino, N., Iida, H., and Ito, M. Molecular cloning and functional analysis of zebrafish neutral ceramidase. J.Biol.Chem., 279: 44012-44022, 2004.

51. Romiti, E., Meacci, E., Donati, C., Formigili, L., Zecchi-Orlandini, S., Farnararo, M., Ito, M., and Bruni, P. Neutral ceramidase secreted by endothelial cells is released in part associated with caveolin-1. Arch.Biochem.Biophys., 417: 27-33, 2003.

52. Dolo, V., D'Ascenzo, S., Sorice, M., Pavan, A., Sciannamblo, M., Prinetti, A., Chigorno, V., Tettamanti, G., and Sonnino, S. New approaches to the study of sphingolipid enriched membrane domains: the use of electron microscopic autoradiography to reveal metabolically tritium labeled sphingolipids in cell cultures. Glycoconj.J., 17: 261-268, 2000.
53. Prudovsky, I., Mandinova, A., Soldi, R., Bagala, C., Graziani, I., Landriscina, M., Tarantini, F., Duarte, M., Bellum, S., Doherty, H., and Maciag, T. The non-classical export routes: FGF1 and IL-1alpha point the way. J.Cell Sci., 116: 4871-4881, 2003.

54. Soldi, R., Mandinova, A., Venkataraman, K., Hla, T., Vadas, M., Pitson, S., Duarte, M., Graziani, I., Kolev, V., Kacer, D., Kirov, A., Maciag, T., and Prudovsky, I. Sphingosine kinase 1 is a critical component of the copper-dependent FGF1 export pathway. Exp.Cell Res., 313: 3308-3318, 2007.

55. Saba, J. D. and Hla, T. Point-counterpoint of sphingosine 1-phosphate metabolism. Circ.Res., 94: 724-734, 2004.

56. Bassi, R., Anelli, V., Giussani, P., Tettamanti, G., Viani, P., and Riboni, L. Sphingosine-1-phosphate is released by cerebellar astrocytes in response to bFGF and induces astrocyte proliferation through Gi-protein-coupled receptors. Glia, 53: 621-630, 2006.

57. Stahelin, R. V., Hwang, J. H., Kim, J. H., Park, Z. Y., Johnson, K. R., Obeid, L. M., and Cho, W. The mechanism of membrane targeting of human sphingosine kinase 1. J.Biol.Chem., 280: 43030-43038, 2005.

58. Heijnen, H. F., Schiel, A. E., Fijnheer, R., Geuze, H. J., and Sixma, J. J. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. Blood, 94: 3791-3799, 1999.

59. Mackenzie, A., Wilson, H. L., Kiss-Toth, E., Dower, S. K., North, R. A., and Surprenant, A. Rapid secretion of interleukin-1beta by microvesicle shedding. Immunity., 15: 825-835, 2001.

60. Pyne S. and Pyne, N. J. Sphingosine 1 phosphate signaling via the endothelial differentiation gene family of G protein coupled receptors. 115-131. 2000. Pharmacol. Ther.

61. Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C. X., Hobson, J. P., Rosenfeldt, H. M., Nava, V. E., Chae, S. S., Lee, M. J., Liu, C. H., Hla, T., Spiegel, S., and Proia, R. L. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J.Clin.Invest, 106: 951-961, 2000.