Communication

Up-regulation of Caveolae and Caveolar Constituents in Multidrug-resistant Cancer Cells*

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Cancer chemotherapy often results in the development of multidrug resistance (MDR), which is commonly associated with overexpression of P-glycoprotein (P-gp), a plasma membrane drug efflux ATPase. It was shown recently that glycosphingolipids are elevated in MDR cells. Sphingolipids are major constituents of caveolae and of detergent-insoluble, glycosphingolipid-rich membrane domains. Here we report that multidrug-resistant HT-29 human colon adenocarcinoma cells exhibit a 12-fold overexpression of caveolin-1, a 21-kDa coat/adaptor protein of caveolae. Similar observations were made in adriamycin-resistant MCF-7 human breast adenocarcinoma cells. Caveolin-2 expression is also up-regulated in MCF-7-AdrR cells, but neither caveolin-1 nor caveolin-2 were detected in MCF-7 cells stably transfected with P-gp. The up-regulation of caveolins is associated with a 5-fold increase in the number of caveolae-like structures observed in plasma membrane profiles of HT-29-MDR cells and with the appearance of a comparable number of caveolae in MCF-7-AdrR cells. A significant fraction (40%) of cellular P-gp is localized in low density detergent-insoluble membrane fractions derived from either HT-29-MDR or MCF-7-AdrR cells. The distribution of recombinant P-gp in stably transfected MCF-7 cells was similar, even though these cells do not express caveolins and are devoid of caveolae. The possibility that caveolae contribute to the multidrug resistance and thus are co-selected with P-gp during the acquisition of this phenotype is discussed.

Although chemotherapy improves long term survival in cancer patients, the treatment often results in the development of tumors that are resistant to most cytotoxic drugs commonly used in chemotherapy, leading to an untreatable and incurable disease (1). Known as multidrug resistance (MDR), this phenomenon may be defined as the ability of cancer cells exposed to a given drug to resist the cytotoxic actions of a broad range of structurally and functionally unrelated drugs. MDR is often caused by overexpression of a plasma membrane ATPase called P-glycoprotein (P-gp) (2). P-gp acts as an energy-dependent drug efflux pump, increasing outward transport of active drugs and thereby decreasing their intracellular concentration and reducing their cytotoxic efficacy. However, additional mechanisms that contribute to MDR have been described (see Ref. 3 for review).

Recent studies have indicated that glucosylceramide accumulates to a major extent in various types of MDR cells (4). Glucosylceramide and other glycosphingolipids are important constituents of detergent-insoluble membrane domains termed DIGs (5) that are enriched also in sphingomyelin and cholesterol (6). DIGs are related in their lipid composition and their insolubility in cold non-ionic detergents to nonclathrin-coated, plasma membrane vesicular invaginations termed caveolae (reviewed in Ref. 7). Caveolin-1, a 21-kDa integral membrane protein, is a major caveolar coat protein (8) that has the ability to engage in complex interactions with other caveolin molecules, as well as other proteins (9). Heterologous expression of caveolin-1 induces the appearance of caveola-like vesicles in cells that normally lack caveolae, e.g. lymphocytes, SF9 insect cells, and transformed fibroblasts (10–12). Caveolae have been implicated in a number of plasma membrane transport processes such as endocytosis (13), transcytosis (14), and cholesterol efflux (15). In addition, caveolae are enriched in signaling molecules, and certain receptor, transducer, and effector proteins are recruited onto caveolae upon cell surface receptor activation (9, 16). The accumulation of glucosylceramide in MDR cells (4) prompted us to examine the possible role of caveolae in multidrug resistance.

EXPERIMENTAL PROCEDURES

Cell Culture—Human colon adenocarcinoma HT-29-wt and HT-29-MDR cells (17) were kindly provided by Prof. I. Cabantchik (Hebrew University, Jerusalem). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. HT-29-MDR cells were maintained in the same medium supplemented with colchicine (300 ng/ml). HT-29-MDR cells were plated in drug-free medium prior to experiments. MCF-7 human breast adenocarcinoma cells, adriamycin-resistant MCF-7 cells (MCF-AdrR), and MCF-7 cells stably transfected with P-gp (BC-19) were kindly provided by Dr. Merrill E. Goldsmith (National Cancer Institute, Bethesda, MD). The MCF-7 and derived cell lines were grown according to published procedures (18, 19).

Isolation of Caveolin-rich Membrane Domains—Caveolin-rich membrane domains were purified from cultured cells as a low density, Triton-insoluble complex, essentially as described (20). The protein content of each fraction was determined according to the modified Lowry procedure (21).

Immunoblot Analysis of Caveolin-1, Caveolin-2, and P-gp—Aliquots taken from each of the sucrose density gradient fractions were separated by electrophoresis on 7.5% or 15% SDS-polyacrylamide gels (for resolution of P-gp and caveolin-1, respectively). Proteins were transferred to nitrocellulose membranes and blocked by incubation for 1 h with 5% skim milk (w/v) in phosphate-buffered saline containing 0.1% Triton X-100. Immunoblot analysis was carried out with monoclonal antibodies to caveolin-1 (clone 2297, Transduction Laboratories), caveolin-2 (clone 65, Transduction Laboratories), or anti-P-gp (C219; Signet), all utilized in a dilution of 1:1000 in the blocking buffer. The blots were then washed extensively and reacted with horseradish peroxidase-linked goat anti-mouse IgG. Bands were visualized by enhanced chemiluminescence using a commercially available kit (Amersham Pharma-
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RESULTS AND DISCUSSION

Up-regulation of Caveolin in MDR Cancer Cells—The specific sphingolipid- and cholesterol-enriched composition of caveolae facilitates their purification on sucrose density gradients together with other detergent-insoluble, low density membrane domains (20, 22). Triton X-100-based lysates were prepared from HT-29 human adenocarcinoma cells and fractionated in a discontinuous sucrose density gradient, and the fractions were analyzed for protein concentration and caveolin-1 immunoreactivity. The distribution of protein along the gradient was characteristically skewed, with most protein concentrated at high density sucrose fractions 8–12 (Fig. 1A). There was no apparent difference between parental HT-29-wt and multidrug-resistant HT-29-MDR cells in either the level or distribution of protein. Caveolin-1 was concentrated in low density fractions 4 and 5 (Fig. 1, B and C). The level of caveolin-1 in HT-29-MDR cells was greatly increased (12-fold) as compared with the parental cell line (Fig. 1, B and C). An even more dramatic up-regulation of caveolin-1 immunoreactivity was observed in multidrug-resistant MCP-7 breast adenocarcinoma cells. In the parental, drug-sensitive cells caveolin-1 expression was undetectable (Fig. 2A, top panel); in contrast, there was a massive caveolin-1-immunoreactive 21-kDa band in the adriamycin-resistant MCP-7-AdR cells (Fig. 2A, middle panel). To test the possibility that the up-regulation of caveolin-1 is a consequence of overexpression of P-gp in the MDR cells, caveolin-1 levels were examined also in MCP-7 cells stably transfected with P-gp (BC-19 cell line). These cells express P-gp levels that are comparable with those found in MCP-7-AdR (Ref. 19; cf. Fig. 4). As shown in Fig. 2A (bottom panel), caveolin-1 was undetectable in these cells. These results indicate that the up-regulation of caveolin-1 in MDR cancer cells might be a general phenomenon in MDR cancer cells and that the higher level of caveolin-1 is not a secondary cellular response to the overexpression of P-gp.

Caveolin-2 is a homolog of caveolin-1 (23). The two proteins are known to be co-expressed in most cell types (24) and are thought to form hetero-oligomers in basolaterally destined caveolae (25). It was therefore of interest to determine whether the up-regulation of caveolin-1 is accompanied by overexpression of caveolin-2. Fig. 2B shows that this is indeed the case; whereas caveolin-2 is undetectable in parental MCP-7 (Fig. 2B, top panel), there is a strong band of caveolin-2 immunoreactive protein in MCP-7-AdR cells (Fig. 2B, middle panel). In contrast, the expression of caveolin-2 in BC-19 cells was too low to be detected (Fig. 2B, bottom panel).

Previous results have indicated that another constituent of caveolae, the glycosphingolipid glucosylceramide, is elevated in various MDR cell lines (4). Examination of glucosylceramide levels in [3H]serine-labeled HT-29 and HT-29-MDR cells revealed that in those colon cancer cells, too, glucosylceramide is significantly elevated (0.15 ± 0.01 versus 0.63 ± 0.15% of total lipid radioactivity in HT-29 and HT-29-MDR cells, respectively). High glucosylceramide levels found in the HT-29-MDR cells were sensitive to 1-phenyl-2-decanoylamino-3-morpholino-1-propanol, an inhibitor of glucosylceramide synthase, which caused a 47–68% decrease in [3H]glucosylceramide levels in the MDR cells, suggesting that the enzyme is constitutively active in these cells.

The fact that three specific constituents of caveolae, namely caveolin-1, caveolin-2, and glucosylceramide, are up-regulated in MDR cells, raised the possibility that acquisition of the MDR phenotype in cancer cells is associated with an increase in the number of caveolae. Electron microscopic examination of plasmalemmal profiles from HT-29-MDR cells revealed the presence of numerous non-clathrin-coated vesicular structures, 50–100 nm in diameter, that are juxtaposed to the plasma membrane and are morphologically indistinguishable from caveolae (Fig. 3A, main panel). In contrast, profiles of HT-29-wt cells typically contained significantly fewer caveolae-like structures (Fig. 3B). Quantitative analysis of multiple plasmalemmal profiles revealed that the HT-29-MDR cells contained over 5-fold more caveolae-like structures as compared with the parental cells (3.5 ± 1.8 versus 0.65 ± 0.7 caveole/10 μm, respectively). Similar results were obtained in MCP-7 and MCP-7-AdR cells. Whereas the parental MCP-7 cells had virtually no caveolae, the MCP-7-AdR cells had 2.4 ± 1.4 caveolae/10 μm. Together with the biochemical data presented above, showing the up-regulation of caveolin-1, caveolin-2 and glucosylceramide, these results indicate that MDR cells have significantly more caveolae and suggest that these structures may play a role in multidrug resistance.

Localization of P-gp in Caveolin-rich Membrane Domains—As in most other cell lines that acquired MDR (1, 26),
Up-regulation of caveolin-1 and caveolin-2 in multidrug-resistant MCF-7 human breast adenocarcinoma cells. Parental MCF-7 cells, multidrug-resistant MCF-7-AdrR cells and BC-19 cells (MCF-7 cells stably transfected with P-gp) were lysed and fractionated by flotation in a discontinuous sucrose density gradient. The fractions were analyzed for caveolin-1 (A) and caveolin-2 (B) immunoreactivity. The locations of the caveolin-1 and caveolin-2 immunoreactive bands (arrowheads) and of the 21-kDa molecular mass marker are indicated. Lane C denotes the position of a caveolin-1 or -2 standard (top and bottom panels) or an aliquot of rat adipocyte membranes (middle panel).

The multidrug-resistant phenotype of HT-29-MDR cells is caused by overexpression of the MDR drug efflux ATPase P-glycoprotein, the product of the human MDR1 gene. Immunoblot analysis of fractions derived from HT-29-MDR cells with antibodies to P-gp revealed that 40% of cellular P-gp is associated with the low density fractions that contain caveoleae (Fig. 4A). The level of P-gp in HT-29-wt cells was usually undetectable. Similarly, 38% of total cellular P-gp was localized in caveolin-rich low density fractions of MCF-7-AdrR cells (Fig. 4B). These data suggest that a significant fraction of cellular P-gp is localized in detergent-insoluble low density membrane domains. To establish whether the fraction of P-gp present in such domains is localized in caveolae proper or in the related membrane domains termed DIGs, we examined the distribution of recombinantly expressed P-gp in BC-19 cells. In these cells, which do not express detectable levels of caveolin-1 and caveolin-2 (cf. Fig. 2), the distribution of P-gp is very similar to that of endogenous P-gp found in MCF-7-AdrR cells, with a significant proportion (24%) of P-gp localized in low density detergent-insoluble membranes (Fig. 4C). Thus, it would seem that the targeting of P-gp to these membrane domains is not dependent on the presence of caveolin or caveoleae. It may therefore be concluded that cellular P-gp is located in at least two distinct membrane compartments. One compartment is detergent-soluble, whereas the other one has the physical properties of DIGs and caveolae, namely detergent-insolubility and low buoyant density. The expression of caveolin is not a necessary condition for targeting P-gp to the DIGs/caveolae fraction.

An interesting issue that requires much further study is the relative contribution of those two distinct P-gp pools to drug efflux and drug resistance in the MDR cells.

Caveolae and Multidrug Resistance—The present results raise the interesting and potentially important possibility that caveolae play a role in multidrug resistance of cancer cells. The up-regulation of caveolar constituents, both protein (caveolin-1 and caveolin-2) and lipid (glucosylceramide), coupled with the greater abundance of caveoleae-like structures, all indicate that MDR cancer cells produce more caveolae. What might be the role of caveolae in MDR? Of the different functions assigned to caveolae, their proposed role in mediating cholesterol efflux might be most relevant in the context of MDR. Fielding et al. (27, 28) have shown that caveolae serve as plasma membrane terminals for the outward transport of cellular cholesterol and thus act to reduce cellular cholesterol levels. Furthermore, caveolin-1 expression is under the control of free cholesterol, acting through the sterol regulatory element-binding protein (SREBP) pathway (29, 30). The SREBP pathway has been implicated in the transcriptional activation of enzymes and proteins involved in biosynthesis or uptake of cholesterol (31). In contrast, SREBP has a suppressive effect on caveolin-1 expression (30). Although the molecular mechanism of free cholesterol efflux through caveolae is not known, it is possible that caveolae (or caveolin) may be capable of mediating or facilitating the export of lipophilic drugs via a similar or analogous mechanism (probably at relatively low efficiency). If caveolae do have such a capability, cells that express higher levels of caveolin (and hence have more caveolae) will have a selective advantage during early stages of drug exposure. This property will therefore be positively co-selected, together with high P-gp expression, in drug-treated cells. This hypothesis is amenable to experimental scrutiny and is currently under study in our laboratory.

It is interesting to note that caveolae and P-gp have been implicated independently in sterol metabolism of normal cells. As mentioned above, caveolae were proposed to constitute a site of cholesterol efflux (27, 28, 32, 33), and sterols were shown to have a strong modulatory effect on caveolin-1 expression via sterol-response elements found within the 5′-flanking region of
were prepared from MCF-7-AdrR (12) and BC-19 cells (14). In agreement with these observations and for critical reading of this manuscript. We are grateful to Prof. I. Cabantchik for providing the HT-29 cells and to Dr. Merrill E. Goldsmith for providing the MCP-7-derived cells.

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