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13. ABSTRACT (Maximum 200 Words)
uPAR (urokinase-type plasminogen activator receptor) is a key player in metastasis of breast cancer cells. We suggest that uPAR, because it is a GPI-anchored protein, must be present in discrete "rafts" in the cell surface to function. Our proposal has two parts. First, we will set up systems in our lab for studying signaling trough uPAR in cultured human breast cancer cells. Second, we will disrupt rafts, and determine whether signal transduction is affected. Our most important advance this year has been in developing new tools for raft disruption. These include sterol analogs such as androstanol and coprostanol. Replacing cholesterol with these analogs allows us to disrupt rafts without depleting total cellular sterol, allowing raft disruption without the other pleiotropic effects that accompany bulk sterol removal. This will be an important tool in later experiments to examine the effect of raft disruption on uPAR-Mediated signaling and cell motility. We anticipate in the next year, we will develop improved methods for detecting uPAR in rafts in cells. We will then determine how the localization of uPAR in rafts governs its deadly activity in metastasis.

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INTRODUCTION

Sphingolipid and cholesterol-rich microdomains called lipid rafts play key roles in signal transduction in normal cells and in transformed breast cancer cells. Proteins anchored in membranes by covalent linkage to glycosyl phosphatidylinositol (GPI; GPI-anchored proteins) are generally highly enriched in rafts. As we have shown, this is because the saturated acyl chains characteristic of GPI anchors fit well into the tightly-packed lipid environment present in rafts. As one key example, the urokinase-type plasminogen activator receptor (uPAR) is a GPI-anchored protein. UPAR, like other GPI-anchored proteins, is known to be enriched in rafts. Activated uPA, bound to the cell surface via uPAR, converts plasminogen to plasmin, allowing degradation of extracellular matrix components and facilitating metastasis. UPAR can also send intracellular signals, activating the MAPK pathway and stimulating cell motility. Thus, uPAR couples matrix degradation and stimulation of cell motility, further stimulating metastasis. Because uPAR is GPI-anchored, it is restricted to the outer surface of the cell. Thus, it cannot contact its intracellular signaling partners directly. It falls in the class of several other GPI-anchored signaling proteins for which this is true. Instead of binding downstream signaling proteins directly, these proteins interact with them indirectly, through mutual association with membrane rafts. Thus, raft lipids serve as the "glue" that holds signaling proteins such as uPAR together with their downstream signaling partners. We have proposed that disrupting rafts, and thus disrupting the interaction of signaling proteins with each other, could be a key means of blunting aberrant uPAR signaling in breast cancer cells, eventually leading to anti-metastasis therapies. In the past year, we have continued our work in identifying novel means of disrupting rafts. We have studied the ability of a series of sterol analogs, similar in structure to cholesterol to inhibit raft formation. As a read-out, we have examined the distribution of GPI-anchored proteins on the surface of cultured cells. Finally, we have examined the protein caveolin, known to be crucial for organizing rafts in a wide variety of cells. We have examined the effects of a caveolin mutant, which is known to be selectively expressed in some breast cancer cells, on raft formation and disruption.

BODY

Task 1. To establish systems for uPAR signaling in breast cancer cells in our lab, and to determine whether uPAR functions observed in other cells are also seen in breast cancer cells. Progress on this Task has been disappointingly slow. We have still had difficulty detecting uPAR cleanly using commercially available antibodies. We started by trying to detect uPAR in MCF7 breast cancer cells by standard immunofluorescence microscopy, using commercially available antibodies (American Diagnostica, Inc). We detected only a very dim, background-like staining. Increasing the concentration of primary or secondary antibodies did not improve specific staining, although non-specific background staining (of similar intensity with or without primary antibodies) was observed as the concentration of secondary antibodies was increased. In an attempt to improve weak staining, we tried secondary antibodies linked to a variety of fluorophores. These included fluorophores of the Alexa series from Molecular Probes, often considered to be more intense than the conventional fluorescein, rhodamine, or Texas red stains. We also attempted to enhance staining using a "sandwich" technique, adding an additional layer of antibodies to amplify the signal. None of these approaches were successful.

We also attempted to detect uPAR by Western blotting, using the same antibodies. All of the cell and tissue sources described above were examined. We were unable to unambiguously detect the ca. 50 kDa uPAR protein, above the considerable background, from any of these. Various

conditions of antibody concentration, blocking, and sample pre-treatment were without effect. Similar results were obtained using nitrocellulose or nylon membranes. Progress on this Task has been further delayed by the unexpected departure of the post-doctoral fellow who had been performing these studies. However, a new fellow will join the lab shortly, and will resume the project. We have recently become aware that other investigators have reported similar difficulties using the commercially available antibodies to detect uPAR. Several investigators have generated antibodies in their labs for this purpose. As detecting uPAR is crucial for the experiments proposed in Task 1 and also in Task 3, we are currently the possibility of obtaining antibodies from other labs for these studies.

It seemed possible that MCF7 cells might contain relatively low levels of uPAR. If so, and if the antibody was sub-optimal, better results might be obtained with different cells. For this reason, we also examined 3 other breast cancer cell lines; MCA-MB-231 (reported to express high levels of uPAR), MDA-MB-435, and SKBR3. We were unable to obtain unambiguous results on any of these lines. Finally, hoping to obtain positive results even if it were not in breast cancer cells, we examined primary HUVEC (human umbilical vein endothelial cells), obtained in collaboration with Dr. Martha Furie (University at Stony Brook, Stony Brook NY), expected to be a rich source of uPAR. Although we detected a dim cell-surface stain in these cells, it was not sufficient for further analysis. As we routinely perform immunofluorescence microscopy, detecting other proteins in other cell types, in the laboratory, our methodology and appears to be adequate for this technique.

Because of these continuing difficulties, we have started taking another approach to this problem, in parallel. We have started to examine other GPI-anchored proteins. Because different GPI-anchored proteins associate with rafts in a similar manner, we expect that initial studies on these proteins will be directly applicable to uPAR, when we have the detection methods for this protein well in hand. Specifically, we have examined the GPI-anchored protein placental alkaline phosphatase (PLAP) as a model. We have started by demonstrating that we could detect transfected PLAP cleanly on the surface of transfected MCF7 human breast cancer cells (Fig. 1). Further studies with PLAP, as a model for uPAR, after raft disruption, are described in the next section (Task 2).

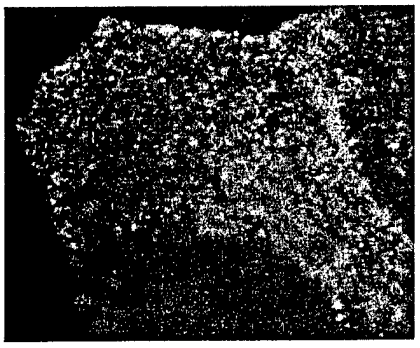

Fig. 1. Immunofluorescence localization of PLAP. MCF-7 cells transfected with PLAP were incubated with anti-PLAP and then with fluoresceinated goat anti-rabbit IgG, and visualized by fluorescence.

Task 2. Disrupt cholesterol and sphingolipid-rich rafts. This is a key goal of our proposal, and much of our effort and much of our progress in the past year have been centered on it. We previously confirmed earlier work showing that depletion of cholesterol with drugs such as methyl beta cyclodextrin (MBCD) could disrupt rafts. A major concern of this method is that cholesterol depletion may have pleiotropic effects on cells. Cholesterol may constitute up to 50% of the total lipid in the plasma membrane on a mole basis. MBCD efficiently removes up to 70-80% of total cellular cholesterol in less than an hour. This occurs because MBCD, a hydrophilic molecule, has a hydrophobic pocket that precisely fits cholesterol. Thus, high concentrations of MBCD added to cells extract cellular cholesterol into the binding pocket, converting it to a soluble, non-cell-associated form that can easily be washed away. Importantly, this treatment is readily reversible. Cholesterol can be pre-loaded into MBCD, and MBCD-cholesterol complexes added to cholesterol-depleted cells can efficiently deliver cholesterol back to the cells, to levels as high or higher than are normally present there. This approach has shown that MBCD treatment is not directly toxic to

cells. Thus, cholesterol can be removed for at least several hours, and then restored by addition of MBCD-cholesterol complexes, with no long-term toxicity to the cells. Nevertheless, cholesterol removal can easily have subtler and more transient effects on a number of cellular processes. In addition to disrupting rafts, then, cholesterol removal with MBCD could easily affect other functions that relied simply on cholesterol mass in the membrane, or on specific interactions of cholesterol itself with individual membrane proteins. This is a concern for two reasons. First, our goal (outlined in the Statement of Work) is to determine the importance of rafts themselves in signaling and metastasis in breast cancer cells. If cholesterol depletion affects signaling through some other means, rather than by disrupting rafts, then further treatments and therapies based on raft disruption might not be relevant. Second, although cholesterol-depleted cells are viable in the short term, it is well-known that cholesterol is essential for long-term health on the cellular and organismal level. Thus, simply extracting cholesterol is unlikely to be a useful means of therapy for breast cancer, even if rafts are disrupted and uPAR signaling is blunted. This is because cholesterol depletion is likely to be harmful to cells for reasons that have nothing to do with rafts. For this reason, we have explored an alternate, though related, method of disrupting rafts. A number of compounds have been identified that are structurally related to cholesterol. Some of these are natural products, and fill the sterol requirement of organisms other than mammals. For instance, the commonly-studied yeast Saccharomyces cerevisiae contains ergosterol instead of cholesterol, while plants contain sitosterol. In each eukaryotic organism, the relevant sterol makes up a similarly high fraction of the total plasma membrane lipid, and performs a similar function. In addition, a number of artificial sterol analogs, similar in overall structure to cholesterol, have also been developed. Work of the London group has shown that some of these can replace cholesterol in enhancing formation of rafts in model membranes (Xu et al., 2001; Xu and London, 2000). Importantly, other sterol analogs have no effect, and some even disrupt rafts formation. That is, lipid mixtures have less tendency to form rafts than they would with no sterol at all. We found that many of these compounds can bind to MBCD like cholesterol. Furthermore, MBCD-sterol complexes can deliver several of these to the plasma membranes of cells form which cholesterol has previously been depleted using MBCD. Thus, it is possible to replace cholesterol with similar amounts of another natural or artificial sterol, that either has the ability to form rafts, or has the ability to disrupt rafts. This is likely to overcome many of the pleiotropic effects of cholesterol depletion, because cells repleted with a sterol analog will have the same sterol mass as untreated cells. By choosing the appropriate analog, it should be possible to replace other essential functions of cholesterol (for instance, interactions with specific proteins (Murata et al., 1995; Thiele et al., 2000)) while still achieving efficient raft disruption. This could effectively block uPAR signaling and other raft-dependent functions, while preserving other essential functions of sterols in membranes.

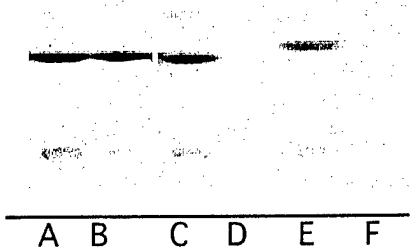

Fig. 2. TLC analysis of liposomes containing: DOPC + DPPC + cholesterol (A) DRMs prepared from those liposomes (B), DOPC + DPPC + coprostanol (C), DRMs from those liposomes (D), DOPC + DPPC + androstanol (E) or DRMs from those liposomes (F).

For this reason, we have begun examining a panel of sterol analogs. For initial characterization, we have examined the raft-forming behavior of these sterols in model membranes. To assay raft association, and the effect of the sterols on rafts, we used the detergent-insolubility assay that we have developed previously (Brown and London, 1998; Brown and London, 2000; Brown and Rose, 1992; Melkonian et al., 1995; Ostermeyer et al., 1999; Shogomori and Brown,

2003). Androstanol and coprostanol were found to disrupt rafts, and were not included in detergent-resistant membranes (DRMs) prepared from model membranes (Fig. 2). By contrast, like cholesterol (Fig. 2), dihydrocholesterol and ergosterol (not shown) were enriched in the DRMs, showing that they associate tightly with rafts. Liposomes contained the raft-forming phospholipid DPPC (which does not char, so is not detected on the TLC plate, but was enriched in the DRMs), the non-raft-forming phospholipid DOPC (present in the starting liposomes, lanes A, C, E, and visible as the loser band, but extracted by Triton x-100 and not visible in the DRMs, lanes B, D, and F) and sterols, either cholesterol (lanes A and B), coprostanol (lanes C and D) or androstanol (lanes E and F). Sterols are visible as the dark upper band. (The faint uppermost band is an unidentified contaminant). Note that although cholesterol is enriched in DRMs prepared from the liposomes (lane B), both coprostanol and androstanol are completely solubilized, and not detectable in the DRMs (lanes D and F), demonstrating their lack of raft association in vitro. We are now poised to be able to add these sterols to cells using MBCD complexes, and to determine the effect on raft integrity in vivo and uPAR signaling.

Another goal in Task 2 is to examine the cell-surface distribution of uPAR and of GM1 before and after raft-disruption. As outlined in the section on Task 1, we are still working to get the uPAR studies up to speed. However, as discussed above, we are establishing the system using another, model GPI-anchored protein, PLAP. Examining cell-surface distribution of raft markers is one of the most powerful and useful ways of determining the integrity of rafts in cells (Harder et al., 1998; Janes et al., 1999; Viola et al., 1999). Most raft markers appear to be relatively uniformly distributed on the surface of resting cells. This is because rafts in these cells are small and highly dispersed. Only during signaling, when raft proteins become clustered together (for instance, through binding to ligand and subsequent dimerization and oligomerization) do rafts become apparent (Harder, 2001). For reasons that are still not completely clear, during signaling events, the small, fairly unstable rafts present on resting cells aggregate together into much larger, stabler raft structures. These are easily visible by light microscopy, using fluorescently-tagged antibodies or other reagents as probes. Two classes of raft markers are the most commonly used for these studies. The first are GPI-anchored proteins, such as uPAR and PLAP. Antibodies directed against these proteins are added to the surface of liver, unfixed cells and allowed to bind to their targets.

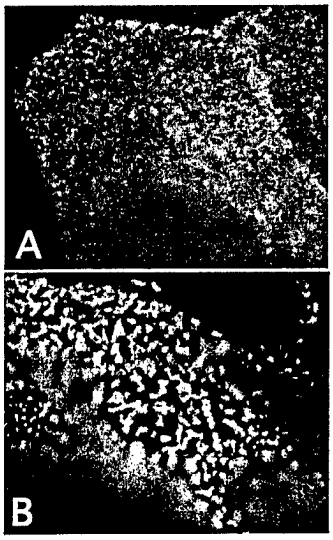

Fig. 3. Effect of antibody-mediated clustering on PLAP distribution. (A) as in Fig. 1. (B), before fixation, rabbit anti-PLAP antibodies and then fluoresceinated goat anti-rabbit antibodies were added to MCF-7 cells transfected with PLAP.

Excess unbound antibodies are then washed away. Next, secondary antibodies (antibodies raised in a different species, that recognize the first or primary antibodies, which themselves recognize the antigen) are added and allowed to bind to the primary antibodies. Importantly, because the secondary antibodies are polyclonal, individual antibody molecules can recognize a variety of different sites on the primary antibody molecule. Furthermore, as each secondary antibody is bivalent, it can simultaneously bind two identical sites, on two different primary antibody molecules. The net effect of these two factors is that secondary antibodies induce clustering of primary antibodies, and – by extension- of the receptor molecule on the surface of the cell that served as the antigen for the primary antibody. This antibody-induced clustering is a useful mimic of physiologically relevant receptor clustering that occurs following ligand binding in cells, and is often used as a convenient model of this clustering. We have established this read-out system in our lab for the GPI-anchored protein PLAP,

as shown in Fig. 3. Panel A shows that in untreated cultured breast cancer cells expressing PLAP, the protein has a uniform distribution on resting cells. After antibody-induced clustering, however, the protein has a dramatically different distribution, as shown in Panel B. It is now observed in large, discrete, ragged clustered patches. These are much larger than would be expected for simple clustering of the protein alone. Instead, the fact that the protein binds rafts causes the small rafts to coalesce together and become stabilized, leading to formation of much larger clustered structures than would be seen otherwise. As expected, when cholesterol was removed with MBCD before treatment with antibodies, much smaller clusters were produced. This will allow us to examine the behavior of protein clustering after repletion of cells with the various sterols that either enhance raft formation, or disrupt rafts, in the model membranes. This is crucial to ensure that the sterols have the same effect on rafts in cells as they do in model membranes, and that GPI-anchored protein distribution is modulated as expected when rafts are disrupted.

We are also examining another species, GM1, as a probe of raft integrity. GM1 is a ganglioside, or acidic glycosphingolipid. (All glycosphingolipids associate tightly with rafts. All are based on ceramide, a raft-associating backbone molecule with long saturated acyl chains that partition well into the ordered raft lipid environment. All glycosphingolipids have one or more sugars linked to ceramide to form a head group. Gangliosides are a subset of glycosphingolipids that contain the negatively-charged sugar sialic acid.) In addition to being tightly associated with rafts, gangliosides serve as binding sites for several bacterial toxins. These toxin proteins bind tightly and specifically to particular gangliosides. Most toxins contain more than one binding site, and thus bind several ganglioside molecules at once. This causes the gangliosides to become clustered, increasing their affinity for rafts still more, as outlined above for GPI-anchored proteins. Because the gangliosides are so highly concentrated in rafts, binding of toxins is a very useful probe for the distribution of rafts on the surface. The most widely-used toxin for this purpose is cholera toxin. Its target ganglioside, GM1, is highly concentrated in rafts. Fluorescently-tagged cholera toxin can be added to cells. It binds specifically to GM1-containing rafts, allowing their visualization with the fluorescence microscope. We have successfully shown that binding of fluorescently-labeled cholera toxin to the surface of cells labels them. We next attempted to further cluster the GM1 (beyond the pentamers formed by virtue of the fact that each molecule of cholera toxin binds 5 GM1 molecules) by further treating the cells with antibodies directed against the toxin. Surprisingly, though, we are so far unable to detect the distinctive raft-like clustered pattern that we have seen for GPI-anchored proteins. Control experiments showed that the secondary antibody (in this case labeled with a red fluorophore to distinguish it from the green labeled fluoresceinated cholera toxin) did bind efficiently to the cells, in a cholera-toxin dependent manner. We do not understand when the toxin did not now assume the distinctive "clustered raft" like appearance shown above for PLAP. We are currently investigating the possibility that the clusters of individual molecules formed by cholera toxin and the primary antibody are not large enough to induce the large-scaled raft stabilization and coalescence seen for GPI-anchored proteins. We will next add a third layer of antibodies, to see whether this will affect the distribution of GM1-labeled rafts.

In the past year, we took one further approach to the important question of raft disruption. That was to examine the role of the membrane protein caveolin. Caveolin forms a coat surrounding the surface of 50-100 nm pits or invaginations in the plasma membrane of cells called caveolae. Caveolin forms high-molecular weight oligomers that associate laterally with each other for form filaments that line the surface of caveolae. Because caveolin has a high affinity for rafts, rafts become concentrated in caveolae. That is, as small, unstable rafts diffuse in the plasma membrane, they tend to remain in caveolae because of their affinity for caveolin. The high concentration of

caveolin in caveolae has the same effect as antibody (or ligand) on causing rafts to coalesce together and stabilizing them. Thus, the entire membrane bilayer in caveolae is probably in the form of a raft. A number of signaling events are concentrated in caveolae (Smart et al., 1999), and they serve as signaling centers at the plasma membrane, concentrating together proteins that need to work together in signaling. Importantly, uPAR is especially highly concentrated in caveolae, reinforcing our hypothesis that association with rafts in the specialized caveolae environment is crucial for uPAR function. Caveolin is often down-regulated in cancers, and caveolae – which depend on caveolin for their formation - suggesting that a down-modulation of signaling (or of regulation of signaling) can enhance tumor progression. However, one particular caveolin mutant (P132L) has been found in 16% of human scirrhous breast cancer lines (Hayashi et al., 2001). We found that P132L lost the ability to associate with rafts, as measured by the fact that it was not enriched in detergent-resistant membranes (DRMs) prepared from cells expressing the protein. This assay is described in detail next. Cells that do not normally express caveolin are transiently transfected with either wild-type caveolin or a mutant – in this case, P132L. Cells are then lysed with buffer containing the non-ionic detergent Triton X-100 on ice. Lysates are then spun at high speed in the centrifuge. The detergent-solubilized material (that does not associate with DRMs) remains in the supernatant, while DRMs (along with cytoskeleton) pellets. Aliquots of the supernatant and pellet fractions are analyzed by SDS-PAGE and Western blotting, probing with anti-caveolin antibodies. The relative amount of the protein in the two fractions shows the affinity of the protein for DRMs and rafts. As shown in Fig. 4A, wild-type caveolin was enriched in the pellet fraction (pel) while P132L (Panel B) was enriched in the supernatant fraction. This suggested that the prevalence of P132L in breast cancer might be related to its loss of raft affinity. Because caveolin, unlike other raft proteins, actively organizes and affects the structure of membrane rafts, it might be necessary for setting up the specific membrane lipid microenvironment required for uPAR signaling. This possibility will be tested in the next year.

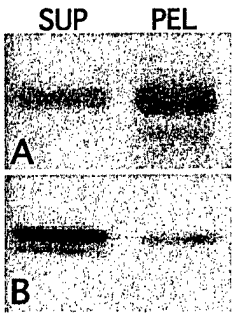

Fig. 4. DRM-association of caveolin and the P132L mutant. MCF-7 cells expressing wild-type caveolin (A) or P132L (B) were extracted with Triton X-100. After centrifugation, detergent-solubilized material in the supernatant (sup) and DRMs in the pellet (Pel) were analyzed by SDS-PAGE and Western blotting.

Task 3. Apply the raft-disruption methods in Task 2 to the uPAR functional assays in Task 1. The difficulty in detecting uPAR with available antibodies, described in the section of the report on Task 1, has prevented us from starting on this task. We anticipate that we will soon this difficulty in the upcoming year, and will be able to make good progress on this task.

KEY RESEARCH ACCOMPLISHMENTS

As detailed in the Body, the most important research accomplishment has been the demonstration that sterol analogs such as coprostanol and androstanol can be used to effectively disrupt rafts, without wholesale sterol depletion from membranes. This should provide a much more directed and specific, but equally effective, method for raft disruption than has been available previously. This will be an important tool in later studies.

REPORTABLE OUTCOMES

The principle investigator gave presentations including the work described here at the following venues:

4/15/02 US Army Medical Research Institute of Infectious Diseases (USAMRIID) Frederick, MD
10/9/02 Johns Hopkins University, Pharmacology Department, Baltimore, MD
1/15/03 Dartmouth University, Pharmacology Department Hanover, NH
3/24/03 Children's Hospital, Boston, MA
6/9/03 Albany Medical College, Albany, NY
9/15/03 St. Louis University, St. Louis, MO

There were no publications or other reportable outcomes of this work in the past year.

CONCLUSIONS

The medical significance of this work remains as described in the original proposal. It is clear that uPA interactions with uPAR play a key role in metastasis, the deadliest feature of breast cancer. Our findings strongly suggest that the presence of uPAR, a GPI-anchored protein, in membrane rafts affects its signaling and its ability to govern cell migration during metastasis. As methods for disrupting rafts are becoming more readily available, the importance of testing the ability of these compounds to inhibit uPAR signaling has never been greater.

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