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This project focuses on angiogenesis, the growth of new blood capillaries, as a potential target for breast cancer therapy and diagnosis. Our approach is based on our past demonstration that many of the signal transducing molecules that mediate the effects of angiogenic mitogens and extracellular matrix molecules are concentrated within specialized cell adhesion sites, known as the focal adhesion complexes (FACs). Over the past year, we began to develop and screen monoclonal antibodies using isolated FACs as an immunogen. Positive hybridoma lines have been identified that label FAC-associated proteins in adherent CE cells and these clones have been isolated. Production of these antibodies and identification of molecules that mediate growth modulation in capillary cells should facilitate development of new angiogenesis inhibitors as well as alternative methods for breast cancer diagnosis.

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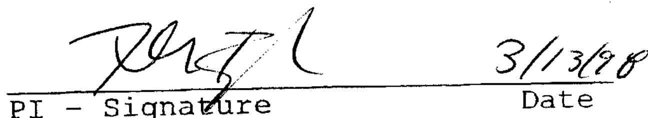

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INTRODUCTION

This project is based on the concept that human breast cancer growth may be suppressed by inhibiting the growth of tumor blood vessels, or what is known as angiogenesis, rather than by targeting the tumor cells themselves. This concept is based on the observation that the cells of solid tumors require continual capillary ingrowth for their own growth and expansion (1). Importantly, while capillary endothelial (CE) cells in tumor microvessels grow very rapidly, normal CE cells are usually quiescent. This differential in CE cell turnover rates therefore provides a potential therapeutic window to selectively prevent tumor expansion without producing generalized cytotoxicity.

Our experimental design is based on past work from our laboratory which revealed that many of the signal transducing molecules that mediate the CE cell growth activation are immobilized on the cytoskeleton at the site of integrin receptor binding within a specialized adhesion structure that is known as the focal adhesion complex (FAC) (2). In addition to integrating signals elicited by binding of growth factors and extracellular matrix molecules, the FAC also provides a path for transfer of mechanical forces across the cell membrane and to the cytoskeleton (3). Mechanical forces transferred across integrins in the FAC drive changes in cytoskeletal organization and cell shape that are required for cell cycle progression (4,5). We recently developed a method to physically isolate intact FACs away from the remainder of the cell and cytoskeleton and have used this approach to demonstrate that isolated FACs retain multiple signal transducing activities in vitro (e.g., protein tyrosine and inositol lipid kinase activities; 2). In the past progress report, we also included data which suggested that the FAC also may play an important role in angiogenesis inhibition. Specifically, we found that the angiogenesis inhibitor, TNP-470, alters tyrosine phosphorylation of FAC proteins.

Thus, the GENERAL GOAL of this proposal is to identify putative molecular targets which could mediate growth stimulation by soluble mitogens or angiogenesis inhibitors in CE cells. The more specific objective is to develop monoclonal antibodies directed against these FAC proteins in order to isolate, sequence, and clone these potential regulatory molecules. Once identified, we would be in a position to determine their role in capillary growth control and thus, to establish a more rational basis for drug design in the field of angiogenesis inhibition. Production of antibodies that recognize growth-associated antigens in CE cells also might form the basis for a more quantitative diagnostic assay for use with tumor biopsy materials.

The specific TASKS proposed under the STATEMENT OF WORK of this grant are:

1. To develop monoclonal antibodies against FAC molecules that are preferentially expressed in growth-stimulated CE cells.

2. To identify antibodies that recognize FAC proteins whose phosphorylation state appears to change in response to treatment with angiogenesis inhibitors.

3. To construct human breast CE cell λgt11 cDNA expression libraries and screen them with these monoclonal antibodies to isolate cDNA clones for the relevant FAC proteins.

4. To explore whether these monoclonal antibodies preferentially detect angiogenic microdomains in histological sections of human breast cancers.

BODY OF THE REPORT

TASK 1:

The major aim of this task is to develop monoclonal antibodies against FAC-related proteins from growth-stimulated CE cells. In the last annual report, we showed preliminary results with a first group of hybridomas. Subsequent work demonstrated that those hybridomas were poor antibody secretors. In addition, they did not produce an specific immunofluorescence staining in CEs or recognize specific proteins in Western blots.

We therefore set out to develop a second set of hybridomas. Intact FACs isolated from FGF-stimulated CE cells using RGD-coated magnetic microbeads were used as antigens (2). Six to eight week old female Balb/c mice were immunized by intraperitoneal injection with approximately 50 ug of FAC in complete Freund's adjuvant. Titers of the antisera were monitored by ELISA. Fifty days later, when the IgM titer began to decrease, the mice were again intraperitoneally immunized with the same amount of FAC protein. The IgG titer increased significantly within two weeks and a final intravenous boost with the same amount of the FAC preparation was administered 5 days later. Three days after the final boost, the spleen was removed and fused with X63-Ag1 myeloma cells following standard procedures (6). Hybridoma growth in selective HAT medium was evident in 40% of the wells after 10 days.
By day 11, the hybridomas were tested using a solid-phase immunoassay that used intact cytoskeleton preparations isolated from adherent CE cells as an antigen, as described in previous reports. Bound antibodies present within hybridoma samples were quantitated using biotinylated secondary anti-mouse antibody in conjunction with an avidin-biotinylated alkaline phosphatase complex. Using this assay, we identified 20 positive hybridoma lines which represented 13% out of the total number of lines that were isolated. A representative set of solid-phase immunoassay positive hybridomas from different culture plates is shown in Fig. 1

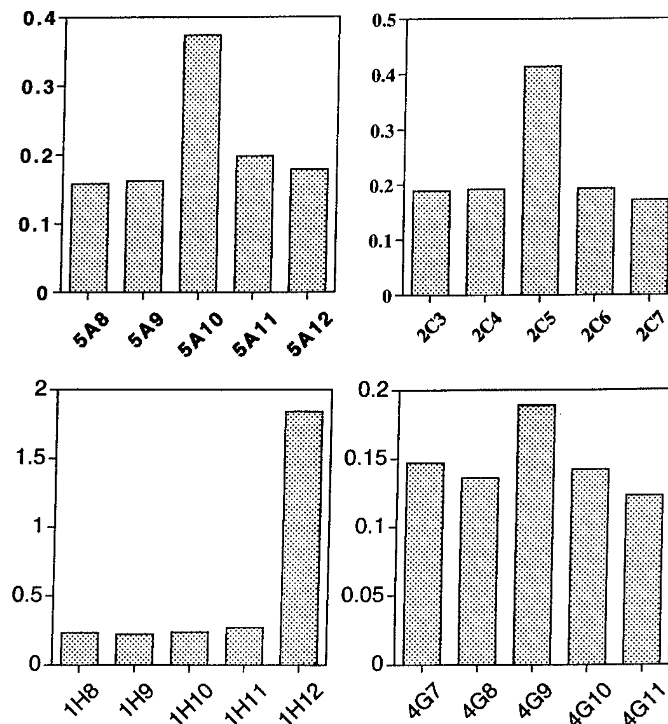

FIGURE 1. Histograms showing results of ELISA assays carried in the screening of new hybridomas. Vertical axis, absorbance (650 nm); Horizontal axis, different hybridoma lines. In this study, supernatants collected from lines 5A10, 2C5, 1H12 and 4G9 exhibited positive cytoskeletal binding activity.

The positive hybridoma lines were then screened using immunofluorescence microscopy in cultured CE cells. Conventional FAC staining approaches are subjective and susceptible to false and confusing positive results due to the great variety in size and shape of individual FACs in adherent cells. To overcome this problem, we adapted a recently described (5,7) micropatterning technique developed in collaboration with George Whitesides (Department of Chemistry, Harvard University) that allows us to make substrates that contained FAC-sized (5 um diameter) circular adhesive islands coated with ECM proteins separated by larger (10 um wide) non-adhesive regions (Fig. 2). The detailed description of the microcontact printing method has been described in previous reports. We used this method to identify antibodies that specifically target to the region of the FAC in adherent, growth factor-stimulated CE cells.

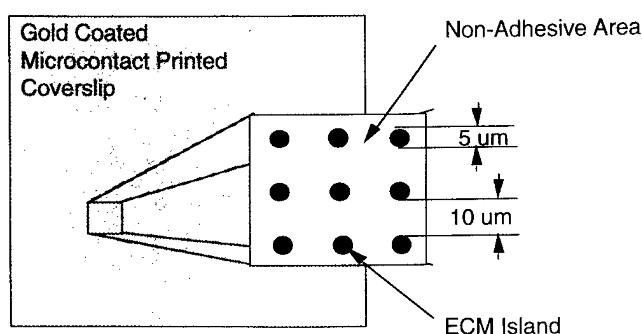

FIGURE 2. Diagram showing the microfabricated substrate containing multiple small (5 um diameter) circular adhesive islands coated with fibronectin. This substrate was used to rapidly screen for antibodies that recognize FAC and cytoskeletal-associated antigens. A single adherent cell spreads over many islands, forming FACs only where they directly contact these matrix-coated circles.

All of the ELISA-positive hybridomas stained positively using this method. Interestingly, patterns of immunostaining were very diverse. Some hybridomas (e.g., 5A10, 2C5), identified proteins that appeared to outline the circular 5 um adhesive islands, in addition to appearing in other regions of the cell (Fig. 3). The FAC-associated staining was similar to that obtained with antibodies against known FAC proteins, such as vinculin, paxillin, and talin as well as total phosphotyrosine (Fig. 3). Other hybridomas identified novel microdomains within the cytoplasm and did not visibly highlight the FAC per se (e.g., 1H12, 4G9) (Fig. 3). Thus, their distribution suggests the existence of cytoskeletal microcompartments that have never been recognized before.

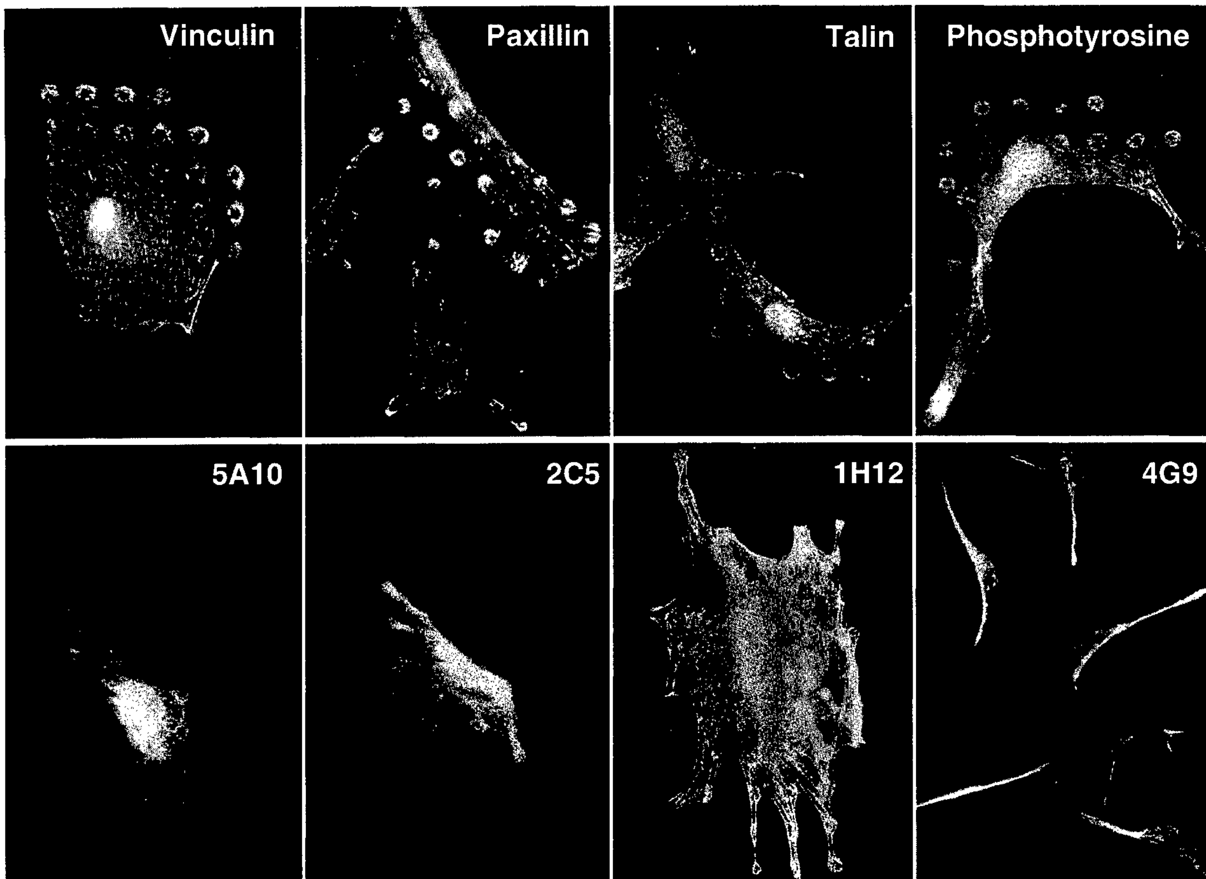

FIGURE 3. Indirect immunofluorescence staining of CE cells attached to microfabricated substrates. The upper row shows the immunostaining of known FAC components: vinculin, paxillin, talin, and tyrosine phosphorylated signaling molecules. The lower row shows immunofluorescence staining with four prototypic hybridoma supernatants developed in the past funding period.

We have started the molecular characterization of the antigens recognized by the hybridomas using metabolic labeling in combination with immunoprecipitation. Semiconfluent growth-stimulated CE cells were incubated in methionine/ cysteine-free medium supplemented with 10% dialyzed fetal calf serum for 2 hours. 30 uCi/ml of [^{35}S]-methionine/cysteine were added and cell cultures were incubated for additional 18 hours to label all the proteins. CSK extracts were dissolved in RIPA buffer (PBS containing 1%NP40, 1% deoxycholic acid, 0.1% SDS) and the remaining insoluble material was removed by centrifugation. The extracts were precleared by 45 minutes incubation with secondary anti-mouse antibodies followed by 30 minutes incubation with *St. Aureus* fixed membranes. Hybridoma supernatants were added to the extracts and incubated overnight at 4°C . Immune complexes were incubated for two hours at 4°C with rabbit anti-mouse IgG and then were precipitated upon addition of proteinA-Sepharose 4B beads. Immunoprecipitated samples were finally washed four times with RIPA, denatured by boiling in Laemmli buffer and subjected to SDS-polyacrilamide gel electrophoresis under reducing conditions. Gels were dried and exposed to X-ray film. Fig.4 displays immunoprecipitations from different experiments with representative hybridoma supernatants. Hybridoma lines 5A10, 2C5, 1H12 and 4G9 recognized specific bands that migrate with approximate and apparent molecular weight of 200, 50, 70 and 100 Kd, respectively.

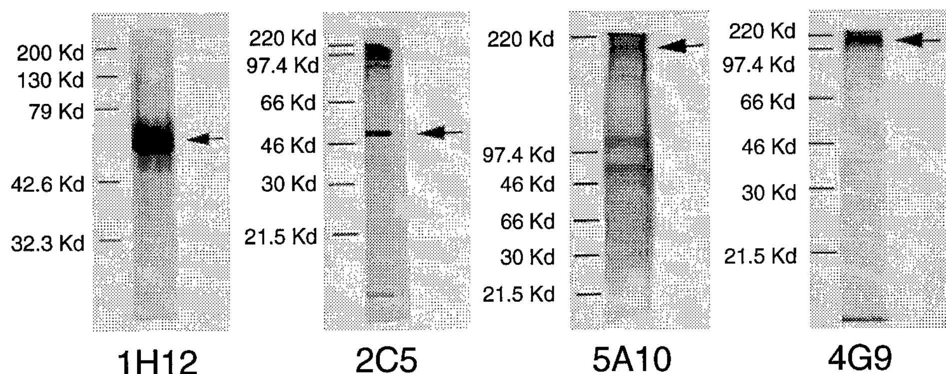

FIGURE 4. Autoradiograph of SDS-PAGE separation of 35S-labeled proteins immunoprecipitated with 4 different hybridoma supernatants (1H12, 2C5, 5A10, and 4G9) that exhibited positive staining by ELISA and immunofluorescence. Arrow indicates the specific protein band recognized by each hybridoma supernatant. The remaining bands were non specific in that they were present in all the immunoprecipitates obtained in a particular experimental condition. Molecular weight markers are shown on the left side of each lane.

Based on these positive results, representative hybridomas were cloned by limiting dilution at least two times. We are now setting up methods to purify monoclonal antibodies from these clones. Specifically, we are currently testing three different types of serum-free media for the adaptation of the representative hybridoma clones. Preliminary results suggest that CCM-1 medium supports efficient hybridoma growth. We are also testing batches of ultra low IgG fetal calf serum for those cases where serum-free media is not sufficient. Once adapted to the appropriate medium, the clones will be grown in a commercially available mini-bioreactor (Unisyn Technologies). This device will allow high density cultivation of hybridomas for high concentration of antibody. Antibodies will be subsequently purified by protein G affinity chromatography.

TASK 2:

No new progress.

TASKS 3 & 4:

We are initiating a second experimental approach using expression cloning to expedite the identification of the antigens that these antibodies recognize. We recently obtained a CE cell cDNA library constructed in a bacteriophage λZAP expression vector. Plaques formed in XL1-blue MRF bacterial cells will be probed with supernatant fluids from the cloned hybridomas. These studies are just getting underway.

CONCLUSIONS

The main objective of this grant is identify specific molecular components of the FAC that mediate CE cell growth stimulation or angiogenesis inhibition. We have developed and begun to characterize our second series of monoclonal antibodies directed against FACs isolated by magnetic microbeads coated with specific integrin ligands from growth factor-stimulated CE cells. They were grouped based on their patterns of immunostaining. The subcellular distribution of the molecules recognized by these antibodies was different from previously defined FAC proteins. Their distribution suggests the existence of different microcompartments in the CSK that have never before been defined. In the next funding period, we hope to use these new antibody reagents we have developed to characterize these novel cytoskeletal components which may be involved in angiogenic regulation. The monoclonal antibodies probes we develop should enhance our ability to identify new angiostatic compounds as well as facilitating breast cancer diagnosis.

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