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The identification of genes, which play a critical role in malignant transformation of the ovarian epithelium, will allow developing molecular tools for detection or intervention of altered activities in ovarian cancer, a cancer, which is often discovered in a late stage and with poor prognosis. Protein kinases such as p21-activated kinases (Paks) are valuable targets for pharmacological intervention. Our project will elucidate alternative molecular mechanisms, present in ovarian cancer cell lines, which lead to hyperactive Pak. The effect of Pak activity on ovarian cell functions and detection of active Pak in tissues are additional goals.

Radiolabeled ovarian cancer cell lines were screened for the presence of activating Pak tyrosine phosphorylation. Our data suggest that this mechanism is not prevalent in these cells. Next approaches will include adenoviral infection of dominant negative signaling molecules and the use of pharmacological inhibitors specific for certain growth factor receptors. Anti-phospho Pak antibody production was not successful, but similar antibodies produced by collaborators will be used for further Pak studies. The influence of Pak on cancer cell proliferation will be delineated with inducible cell lines. Increased knowledge about Pak signaling in cancer is an important step towards Pak inhibitor development.

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INTRODUCTION:

P21-activated kinases (Paks) play essential roles in the regulation of actin cytoskeleton dynamics and nuclear gene transcription. Recently, Paks have been linked to transformation by affecting anchorage independent growth and invasiveness, as well as by participating in signaling pathways emanating from EGF receptors. Hyperactive, deregulated Pak kinase activity was detected in several cancer cell lines (1). While the upstream regulator Rac3 was found to be hyperactive in breast cancer cell lines, the molecular mechanism of Pak activation in ovarian cancer cell lines was not dependent on Rac3 and remains unidentified. We hypothesized that alternate mechanisms of Pak activation in ovarian cancer cells exist and started to pursue several approaches to identify these signaling proteins, to elucidate Pak function in ovarian cancer cells and to detect hyperactive Pak in cells and tissues.

BODY:

Pak hyperactivity in ovarian cancer cell lines

We have shown previously that ovarian cancer cells contain hyperactive Pak but not active GTPases such as Rac1, 3 or Cdc42. Experiments to detect the activation state of endogenous TC10, a GTPase homologous to Cdc42, in ovarian cancer cells were not feasible due to the poor performance of the only antibody available (2). A recent publication described Rho GTPase-dependent tyrosine phosphorylation and activation of Pak in v-ErbB-transformed fibroblasts (3) as well as tyrosine phosphorylation and activation of Pak by the non-receptor tyrosine kinase Etk (4). Furthermore, a connection between Pak and the tyrosine kinase Abl was reported (5). We decided to pursue this new avenue first on our search for alternative mechanisms of Pak activation. Ovarian cancer cells with active Pak (Ovcar3, Skov-3) and normal immortalized ovarian cells (Gayton) were radiolabeled with ^{32}P-phosphate overnight and lysed in protease- and tyrosine phosphatase inhibitor-containing lysis buffer. Pak was immunoprecipitated with polyclonal anti-Pak antibody prepared by us (6), subjected to SDS-PAGE and transferred to nitrocellose, and analyzed by autoradiography and Western blotting. While several Pak isoforms were detected in the immunoprecipitated sample, only one band at 75 kDa showed incorporation of tyrosine. This isoform is presumably Pak4 or 5 according to the molecular weight. Pak tyrosine phosphorylation seemed not to cause Pak activation, since in-gel kinase assays showed only activation for the 62 and 67 kDa Pak isoforms.

We proposed to investigate the intracellular signals leading to Pak activation in ovarian cancer cells by introducing dominant negative signaling molecules into these cells. Ovarian cancer cells are relatively difficult to infect with high efficiency using lipid-mediated transfer. We decided to establish an adenovirus-based infection system, which would allow infecting 90-100% of the cells with low toxicity. Different control viruses encoding the green fluorescent protein GFP were produced. We used Fiber 5 or Fiber3 containing adenovirus, choosing either recombination in HEK293 or in E. coli. After infection of ovarian cancer cells using different conditions and virus concentrations, infection efficiency was determined by FACS analysis. In light of our results we decided to establish an adenovirus Fiber5 system. We started to clone the active GTPase-binding domain PBD, the Pak inhibitory domain PID, the control mutation PID L107F and several dominant negative GTPases into the shuttle vector. Since a Rho-dependent pathway of Pak activation was proposed (3), we will also include the Rho-binding domain RBD.

We will infect ovarian cancer cells with these constructs as soon as high titer viruses are produced, and analyze their effect on Pak activation.

Pak-initiated signaling cascades leading to growth

To achieve this aim we will generate inducible HeLa-Tet cell lines expressing various Pak constructs. We spent considerable time and effort on production and testing of the anti-phospho Pak antibody and have just begun cloning into the pTRE vector. Instead of establishing cell lines with dominant negative and constitutively active full-length Pak, we decided to use GFP-tagged Pak carboxyl terminus (constitutively active), GFP-tagged Pak inhibitory domain (kinase inhibitory) and an inactive point mutation of this domain, as well as a myc-tagged wildtype construct. GFP versions of full-length Pak seem to exhibit altered functions, which we would like to avoid. Furthermore, expression of the PID will inhibit all three isoforms of Pak at the same time. The constructs will be sequenced and expressed in HEK293 cells to confirm their activation status. Kinase activity will be assessed after epitope tag immunoprecipitation by using $p47^{phox}$ as substrate.

Anti-phospho Pak antibodies

The GTPase-Pak activation model predicts that binding of the active form of the GTPase to the Pak CRIB domain dissociates the regulatory from the catalytic domain, thereby relieving kinase inhibition. The Pak activation loop becomes available for autophosphorylation, followed by multiple autophosphorylation events in the amino terminal regulatory domain (7, 8). Since the phosphorylation of a threonine in the activation loop is the first step in the activation process, we chose this site for antibody generation. To generate anti-phospho Pak antibodies we decided to use a fusion protein approach that might result in antibodies with higher sensitivity. The phosphopeptide CEQSKRST(P)MVGTPYC, derived from amino acid residues 419-429 of human Pak1 was coupled to the carboxyl terminus of His6-DHFR via PCR. The construct was expressed in *E.coli* and affinity purified on nickel beads (Talon). The fusion protein was injected into rabbits and the resulting antisera were purified by Protein A sepharose chromatography. Affinity purification of the sera was achieved by passage over a column containing non-phosphorylated peptide, followed by an antigen phosphopeptide column. Bound sera was eluted with 0.1 M glycine, pH 2.5 and immediately neutralized by addition of 1 M Tris, pH 9.0. After dialysis against phosphate-buffered saline the protein concentration was determined. Screening of the antibody was done using overexpressed wildtype and constitutively active Pak as well as by *in vitro* GTPγS-loading of wildtype Pak containing lysates. Additionally, neutrophils were isolated from human blood and stimulated with the chemoattractant fMLP. Stimulated neutrophils show consistently high levels of endogenous, transiently activated Pak 1 and 2, as we have demonstrated previously (6). Unfortunately the antibody showed similar detection of wildtype, inactive Pak and active Pak when Pak was overexpressed and failed to detect active endogenous Pak on Western blots.

During our studies we became aware of the generation of a similar anti-phospho Pak antibody produced to the same Pak activation site. Chernoff J. et al. described that this antibody was not absolutely specific for Pak, recognizing other protein kinases with similar activation loops (i.e. Mst1), but that exogenous active Pak was recognized (9). We obtained this antibody

and tested it on stimulated neutrophil preparations. While Pak was clearly activated in this process as shown by in-gel kinase assays, we could not detect the activation of endogenous Pak. Just recently two more groups prepared anti-phospho Pak antibodies (10, 11), both generated in rabbits to a very similar peptide sequence as used by us. We received one of these antibodies and will begin testing soon. Our present goal is to continue our studies with the most promising antibody and to evaluate its performance in immunofluorescence with fixed cells before we will start to analyze tissue samples.

KEY RESEARCH ACCOMPLISHMENTS:

- Establishment of an adenovirus Fiber5 system and determination of its efficiency in ovarian cancer cells.
- Determination of Pak tyrosine phosphorylation in ovarian cancer cells and normal ovarian cells.
- Generation of anti-phospho Pak antibody with limited performance. Collaboration with several research groups to test different anti-phospho Pak antibodies.

REPORTABLE OUTCOMES:

This award just started and additional work has to be accomplished prior to publication or presentation at meetings.

CONCLUSIONS:

An alternate Pak activation mechanism involving tyrosine phosphorylation has been described since our proposal was submitted and funded. The data concerning Pak tyrosine phosphorylation are not conclusive, reporting either activation or inhibition (Abl). Additionally, the potential tyrosine residue(s) have not been identified. We conclude that tyrosine phosphorylation of one Pak isoform might occur in ovarian cancer cells, but seems not to affect the activity of the kinase. Since overexpression of EGFR, HER2 and HER4 have been shown in ovarian cancers and Pak activity might be correlated to these events, the use of specific pharmacological inhibitors will be included in our studies.

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