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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Purpose: To test the working hypothesis that SHP-1 is essential for controlling growth and differentiation of mammary epithelial cells and that its dysregulation contributes to the development of breast cancer.

Scope: To biochemically and functionally characterize SHP-1 in human breast cancer cell lines and to define its biological function in normal epithelial cells.

Major Findings: We have shown that SHP-1 associates with the EGFR in an EGF stimulation-dependent manner. In addition, we have found that SHP-1 localizes to the lipid rafts. Moreover, our data indicate a functional difference between rafts- and non-rafts-associated fractions of SHP-1. While most of the subcellular localization studies have been performed in hematopoietic cells, in experiments using human breast cancer cells, we have observed that limited amounts of SHP-1 localize to lipid rafts before and after EGF stimulation. In addition, we have obtained additional data showing that a transgenic mouse expressing SHP-1 under its own hematopoietic promoter can partially rescue the *motheaten* phenotype, which has helped to start the development of a new mouse model.

Significance: We expect to gain a better understanding of SHP-1's role in epithelial cells and thereby to learn how a dysregulated SHP-1 is potentially involved in the onset/progression of breast cancer.

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INTRODUCTION

While a strong correlation between the development of breast cancer and expression of certain protein tyrosine kinases (PTKs), such as members of the ErbB family of receptor kinases or the cytoplasmic c-Src kinase, has been observed, little is known about the role of protein tyrosine phosphatases (PTPs) in breast cancer. We have hypothesized, that PTPs would balance the PTK activities and thereby counteract their tumor-promoting actions or unbalanced PTPs could be tumor-promoting themselves. SHP-1 is a cytoplasmic tyrosine phosphatase expressed exclusively in epithelial cells and the hematopoietic lineage. We chose to study SHP-1 as a possible mediator in the onset/progression of breast cancer for the following reasons: (i) In the hematopoietic system, the role of SHP-1 as a negative regulator has been well established. It is conceivable that SHP-1 has a similar role in epithelial cells, and its dysregulation could contribute to neoplasms arising in breast epithelial cells. Biochemical and functional characterization of SHP-1 in breast epithelial cells were being addressed as part of Tasks 1 and 2. (ii) In our preliminary studies, mice that lack one of the wild type SHP-1 alleles have a high incidence of breast tumors, suggesting a role for this phosphatase in the onset/progression of breast cancer. This hypothesis is being addressed in Tasks 3 and 4.

BODY

Task 1 and 2 (Characterization and defining the function of SHP-1 in human breast cancer lines and normal epithelial cells)

To biochemically characterize SHP-1 in epithelial cells and in particular in human breast tumor cell lines, we analyzed a panel of human cell lines but concentrated most of our efforts on the cell line MDA-MB 468. In our preliminary analysis, we had shown that this cell line expresses SHP-1. Moreover, this cell line expresses the Epidermal Growth Factor Receptor (EGFR), but no detectable levels of other members of the ErbB family (1). Thereby, it provides a system where a potential interaction between SHP-1 and the EGFR can be studied without the complication of generating heterodimers between the EGFR and other members of its family.

We have shown that upon EGF stimulation, SHP-1 associates with several tyrosyl phosphorylated proteins, one of which co-migrates with the EGFR suggesting an association between SHP-1 and the EGFR. It is interesting to note that the time course of overall EGFR tyrosyl phosphorylation differs from the time course of SHP-1 associated EGFR. While the majority of phosphorylated EGFR associates with SHP-1 immediately following stimulation with EGF (1 min and 5 min.) and decreases after 5 min., the tyrosyl phosphorylation of the total EGFR peaks between 5 and 10 min, and is sustained up to 20 -30 min. following stimulation. This difference emphasizes that the observed association between SHP-1 and the EGFR is specific and does not reflect a general association of SHP-1 with tyrosyl-phosphorylated proteins. During the course of these studies, other groups have also shown that SHP-1 binds inducibly to the EGFR via its SH2 domains (2). In addition, it has been reported that *in vitro* the EGFR is a substrate for SHP-1 (3) and that co-expression of SHP-1 with the EGFR leads to decreased tyrosine phosphorylation of the EGFR, indicating that the receptor could be also be a substrate for SHP-1 *in vivo* (4). We are currently addressing this question using substrate-trapping mutants of SHP-1 using cellular fractions enriched for SHP-1 (see below).

As described in the previous scientific report, we had encountered problems when we originally attempted to purify substrates of SHP-1 using total cellular lysates. To enrich for SHP-1 before purification, we have started to focus our characterization of SHP-1 on its localization to specialized membrane microdomains, the so-called lipid-rafts. Recently, the importance of the subcellular localization of the involved proteins has been re-emphasized for early signaling events. In particular, the critical role of lipid rafts has been recognized [reviewed in (5-8)]. Cell membranes are composed of proteins and lipids, such as cholesterol and various glycophospholipids and sphingolipids that form microdomains within the membrane. Based on their biophysical properties, glycophospholipids tend to display a mobile fluid phase, whereas sphingolipids show a more tightly packed higher organization [reviewed in (5)]. Moreover, gaps between the fatty-acyl chains of the sphingolipids are filled with cholesterol, thereby forming a closely-packed lateral lipid cluster, the so-called lipid rafts, in an unsaturated glycophospholipid environment [reviewed in (8,9)]. Due to their biophysical properties, these cholesterol/sphingolipid rafts are insoluble in non-ionic detergent at 4°C and can be isolated as low-density complexes in sucrose gradients. They have also been referred to as detergent-insoluble glycolipid-enriched complexes (DIGs) (10), low-density Triton-insoluble fraction (LDTI) (11), or glycolipid-enriched membrane domains (GEMs) (12). Since during the last years a number of studies have focused on lipid rafts and their role in early TCR-signaling [reviewed in (13-15)], we decided to also use T cells for our initial studies and to optimize the conditions for rafts isolations and characterization. For example, several key players in early signal transduction pathways downstream of the TCR, such as the ζ chain of the TCR/CD3 complex, Lck, Fyn, ZAP-70, Shc, LAT, SLP-76 and PLCγ1, have been shown to localize either constitutively or upon stimulation to the rafts fraction (12,16-18). However, SHP-1 has not been analyzed for its subcellular localization.

Using the BYDP T cell hybridoma line, a pre-TCR line and primary thymocytes, we have now shown that about 30-40 % of total SHP-1 is localized to the rafts fraction before and after TCR plus CD4 stimulation. We have also generated fusion proteins between SHP-1 and the Green Fluorescence Protein (GFP) and have shown localization of SHP-1-GFP to lipid rafts using confocal microscopy. Interestingly, we have observed that the rafts-associated fraction of SHP-1 is hypo-phosphorylated compared to the non-rafts fraction in response to TCR/CD4 stimulation. This is not due to an inaccessibility of SHP-1 since treatment with the tyrosine phosphatase inhibitor pervanadate causes phosphorylation of SHP-1 in the lipid rafts. Using the cholesterol-depleting drug methyl-β-cyclodextrin (MβCD), we have shown that induced tyrosine phosphorylation of the non-raft associated fraction of SHP-1 is still raft-dependent. Taken together, our data suggest functional differences between the raft-associated and the non-raft-associated fractions of SHP-1. We are currently addressing the mechanism of rafts localization of SHP-1 using mutants of SHP-1 fused to either an HA tag (for biochemical analyses) or GFP (for confocal microscopy). Our preliminary data suggest that a C-terminal peptide of SHP-1 is, at least partially, mediating this subcellular localization. A manuscript describing the results derived from this study is in preparation.

We are also in the process of addressing the localization of SHP-1 to lipid rafts in epithelial cells. Our initial experiments have shown that a limited amount of SHP-1 localizes to lipid rafts in human breast tumor cell lines before and after EGF stimulation. However, we are still in the process of optimizing the biochemical purification of lipid rafts in epithelial cells since

due to the presence of caveolae, the membrane composition of epithelial cells differs dramatically from the composition of lymphocytes. We expect that better knowledge of the subcellular localization of SHP-1 will enhance the possibility to find binding partners as well as substrates of SHP-1 in epithelial cells. It will be informative to know whether the indicated functional differences between raft-associated and non-raft-associated fraction of SHP-1 observed in lymphocytes will be present in epithelial cells. We expect that results obtained from these studies will provide indications about the place of action for SHP-1, potential up-stream players, such as kinases phosphorylating SHP-1, SHP-1's localization and regulation through other proteins and overall help to gain a better understanding of SHP-1's mechanism of action in epithelial cells.

Task 3 (Defining the biological function of SHP-1 in normal epithelial cells)

We had proposed to generate a transgenic mouse expressing SHP-1 under the control of its hematopoietic promoter. As described in the previous progress reports, we obtained two female founder mice carrying the transgene. However, only one of the mice delivered offspring carrying the transgene. By further breeding, we generated a stock of these transgenic mice. Mice carrying the transgene are viable and, at least based on what we have observed so far, seem normal.

One of the reasons, we had generated this transgenic mouse, was to cross it into the *motheaten* background with the hypothesis to thereby generate a partially rescued *motheaten* mouse, which would allow us to study SHP-1-deficient epithelial cells in an otherwise "normal" background. We observed that *me/me* mice carrying the transgene live for up to 10 weeks compared to the average *me/me* life span of 3-4 weeks. They transgenic *me/me* mice eventually die of the same macrophage-induced symptoms as the non-transgenic. We believe that the transgene might be expressed in a mosaic pattern in a subset of the hematopoietic cells, not uncommon for transgenic mice, and the non-expressing cells expand until they overtake and cause the death.

Since the average life span of these transgenic *me/me* mice still is only 10 weeks and therefore to short to monitor for the development of breast cancer, we are now developing a new mouse model system that does not rely on the rescue of the *motheaten* phenotype but uses tissue-specific expression of dominant negative mutants of SHP-1.

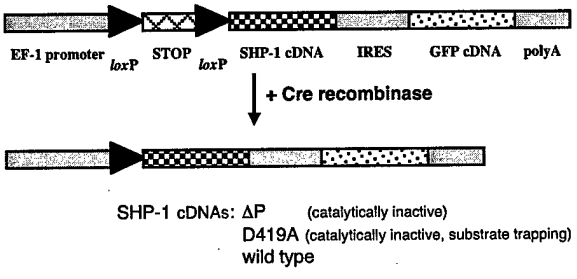

Fig. 1: Transgenic construct to generate mice expressing dominant negative mutants of SHP-1 (ΔP and D419A) in a tissue-specific manner. Depending on the results obtained from these mice, wild type transgenic mice expressing wild type SHP-1 will also be generated.

We have generated a transgenic construct, in which the promoter [elongation factor 1α (EF-1α)] and the SHP-1 cDNA were separated by a well described tight STOP cassette with flanking *lox*P sites (19,20) preventing expression of SHP-1 (Fig 1). Upon Cre-mediated recombination of the *lox*P sites, the STOP cassette will be excised causing the SHP-1 cDNA and the EGFP gene (expressed

as a bi-cistronic message) to be placed adjacent to the EF-1α promoter, which directs ubiquitous expression. The exogenous SHP-1 protein has a FLAG tag to distinguish it from endogenous SHP-1. We chose this approach instead of using a transgenic construct directing expression constitutively to the mammary tissue, since it will allow us to eventually test not only the mammary epithelial cells for an effect of dominant negative SHP-1. Instead, we will be able to assess the role of additional tissues (such as the T cell or the macrophage lineages) in breast cancer promotion by crossing the transgenic mice with the respective Cre-expressing mice.

We have created constructs carrying wild type SHP-1, the deletion mutant ΔP or the substrate-trapping mutant D419A. We will initially generate two transgenic mice using the two dominant negative constructs. We chose these two mutants of SHP-1 since they might have different biological functions due to their ability/inability to trap substrates. Potentially different effects on tumor promotion could give insights into the underlying mechanisms. Mice carrying the transgenes will be crossed into backgrounds expressing the Cre recombinase in breast epithelium, such as MMTV-Cre for the hormone-responsive epithelial lineage (21), and mice will be observed for the development of breast tumors. We will also backcross these transgenic mice into the original *motheaten* backgrounds (C3HeB/FeJLe-*a/a* and C57BL/6J) since they differ in their prevalence for specific tumors. In addition, we will cross these mice into other breast cancer models, such as the MMTV-c-Src or MMTV-EGFR transgenic mice that are currently generated by Dr. S. Parsons' laboratory at the University of Virginia. In these crosses, we will test for a co-operative effect such as earlier on-set/higher incidences of breast cancer in triple transgenic mice (SHP-1 ΔP or D419A, MMTV-Cre together with MMTV-c-Src or MMTV-EGFR) compared to the individual transgenic mice.

Task 4 (Analysis of breast tumors in *me/+* mice)

In our preliminary studies, we had observed that retired *me/+* female mice display an unusual high frequency of breast tumors. Analysis of a larger mouse population confirmed the high incidence of breast tumors with the earliest onset at 11 mo. of age. At the age of 19 mo. >50% and by 24 mo. >90% of the mice show breast tumors (Fig. 2). In the control population of +/+ mice of the same C3HeB/FeJLe-*a/a* strain, we have not observed any breast tumors until the age of 16 mo. and the overall incidences are <50%. Although we were ensured by Jackson Laboratory, where we obtained the original breeder mice from, that these mice were MMTV free, we confirmed this by checking for the presence of MMTV-specific superantigens (22,23) and found that these mice indeed do not delete the T cell receptor Vβ14 chain, which indicates the absence of active MMTV.

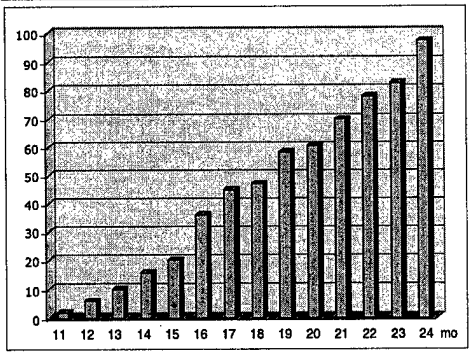

Fig. 2: Percentage of retired female (*me/+*) breeder mice developing breast tumors (n=52).

Key Research Accomplishments

- SHP-1 associates with the tyrosyl phosphorylated EGFR in an EGF stimulation-dependent manner. (Task 1)
- SHP-1 localizes to lipid rafts. Indication that raft-associated and non-raft-associated fractions of SHP-1 show functional differences. Preliminary understanding of the mechanism that causes rafts-localization of SHP-1. (Task 1 and 2)
- Generation of retroviruses that encode wild type and mutant forms of SHP-1 together with GFP allowing identification of infected cells. 60-90 % of epithelial cell population can be infected. (Task 2)
- Generation of transgenic founder mouse carrying cDNA for SHP-1 under the control of its hematopoietic promoter. Cross of transgene into *motheaten* background. Prolonged lifespan in transgenic *motheaten* mice compared to non-transgenic. (Task 3)
- Increased frequency of breast tumors in C3HeB/FeJLe-*a/a* female *me*/+ mice compared to +/+ mice is observed. Absence of MMTV in breast-tumor developing population. (Task 4)
- Development of new mouse model for SHP-1 and its role in breast cancer started. (Task 4)

Reportable Outcomes ----

- U. Lorenz and G.M. Calabrese, Involvement of the Tyrosine Phosphatase SHP-1 in the Development of Breast Cancer, Era of Hope Meeting, Atlanta 2000
- U. Lorenz, G.M. Calabrese and V.C. Johnson, Involvement of the Tyrosine Phosphatase SHP-1 in the Development of Breast Cancer, Era of Hope Meeting, Orlando 2002
- V. Johnson, G. Calabrese and U. Lorenz. Subcellular localization of the tyrosine phosphatase SHP-1. Manuscript in preparation.

Conclusions

During the funding period, we have shown that SHP-1 participates in the signal transduction pathway downstream of the EGFR. Since members of the ErbB family of receptor kinases have been shown to play a role in the development of breast tumors, our findings suggest that SHP-1 might also play a role in this process. In addition, we have obtained data about the localization of SHP-1 to the lipid rafts that indicate a functional difference between rafts-associated and non-associated fractions of SHP-1. While these studies have initially been performed in T cells and primary thymocytes to optimize the experimental conditions, we expect to gain a better understanding of SHP-1's mechanism of action from similar studies in epithelial cells. In fact, in preliminary studies using human breast tumor cell lines, we observed a limited amount of SHP-1 in the lipid rafts before and after EGF stimulation.

In addition, we have created a transgenic mouse carrying a gene for SHP-1 under its hematopoietic promoter. Although this mouse shows only low expression levels it is able to partially rescue the *motheaten* phenotype with respect to longevity upon crossing into the

motheaten background thereby supporting our initial hypothesis that wild type SHP-1 expression in the hematopoietic system can overcome the *motheaten* phenotype. Based on the results obtained from this study, we have designed a new mouse model that we expect to better address the role of SHP-1 in the development of breast cancer. This model will allow us to not only analyze the role of the epithelial cells in this process but to also include the role of surveillance by the immune system.

Finally, we have confirmed that the incidence of breast tumors in the retired *me*/+ female breeder mice is indeed increased compared to their +/+ littermates and that is not due to an MMTV infection.

"So what": In our original grant application, we had proposed as a working hypothesis that SHP-1 is essential for controlling growth and differentiation of mammary epithelial cells and that dysregulation of SHP-1 contributes to the development of breast cancer. Based on the systems we have set up and the reagents we have generated, we believe to have the necessary tools to gain a better understanding of SHP-1's role in epithelial cells. We expect not only to deepen our knowledge of SHP-1's role in epithelial cells but also to learn how a dysregulated SHP-1 is potentially involved in the onset/progression of breast cancer. Moreover, the knowledge of SHP-1's dysfunction and its consequences in certain breast tumors might allow us to use it as a diagnostic and/or a prognostic marker. This might also have implications for possible future therapies.

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