

Final report ESMO Translational Research Fellowship 2010 -2011

"Molecular and genetic analysis of stromal fibroblasts in prostate cancer"

Michalis Karamouzis

Host Institute Department of Biological Chemistry, Medical School, University Of Athens



Rationale and Aim

Contrary to the conventional notion that views carcinogenesis as a cell-autonomous process accumulated evidence attributes increasing significance to the role of stroma, and especially the cancer associated fibroblasts for the development of a malignant tumor. Thus, according to current notion stromal fibroblasts receive signals from the malignant epithelial cells, change their expression profile (and occasionally their genetic content) and enter a cancer-associated state at which they change the epithelial cells' microenvironment, and elicit other signals that result in the promotion of tumor growth. Thus, a network of reciprocal interactions is established between the epithelial and the fibroblastic stromal component of malignant tumors (1). Besides their dynamic properties, stromal fibroblasts acquire certain morphological characteristics, such as increased mitotic indices and atypia that clearly differentiate them from their normal counterparts. At the subcellular level, cancer associated fibroblasts (CAFs) accumulate mutations and display loss of heterozygosity at specific loci that are in the close vicinity of tumor suppressor genes (1). Among them, p53 and PTEN tumor suppressor genes were found to exhibit frequently LOH in stromal fibroblasts from prostatic and breast cancers, and at least in the second these events were mutually exclusive (2,3). Such analyses have been performed in primary human tumors by micro-dissection of the CAFs and their isolation from the epithelial (neoplastic) component of the tumors.

The importance of tumor stroma in carcinogenesis has been exemplified by the fact that isolated CAFs from prostatic tumors, but not from normal tissue, can cause malignant transformation of immortalized prostate epithelial cells (4). Thus, these CAFs elicit potent oncogenic signals. Furthermore, it has been shown recently that ablation of p53 from stromal fibroblasts renders



tumors more aggressive while restoration of its activity delays tumorigenesis, supporting the notion that the profile of tumorigenesis can be altered by modulation of the stromal fibroblasts (5).

Our laboratory, based on an experimental model system we have developed, has undertaken an effort to identify genes that are associated etiologically with the transition of stromal fibroblasts into their cancer – associated state. This model is based on the comparison, by microarray analysis or differential display PCR, between the expression profile of cancer cells cultured in vitro versus the same cells growing in nude mice or SCID mice.

Functional assays have confirmed that at least three genes behave as such, being the endoplasmic reticulum proteins ERp29, KDEL receptor 3 (KDELr3), and the cyclin –dependent kinase inhibitor p21/waf1 (6,7). These genes undergo differential regulation, p21 in the fibroblasts and ERp29 and KDELr3 in the epithelial-neoplastic cells, when cancer cells interact with the stromal fibroblasts and can modulate the efficacy by which this interaction proceeds. Thus, it is conceivable that by modulation of the expression/activity of certain genes in stromal fibroblasts the profile of carcinogenesis will be altered, therefore providing a target for the development of novel cancer therapeutics. The significance of such approach is based on the fact that considering that in general CAFs represent normal –not neoplastic – cells they may exert a uniform response against therapy, devoid of heterogeneity due to the acquisition of mutations like those accumulating into the malignant cells.

Experimental design

Based on the aforementioned - briefly stated - findings, we propose to perform molecular and genetic analyses of stromal fibroblasts from prostatic carcinoma and to explore the feasibility to develop novel therapies targeting the fibroblastic component of prostate cancer. Furthermore, the diagnostic and prognostic significance of the stromal fibroblasts will be assessed. Briefly, the objectives of our study are the following:

Allelotype analysis of prostatic fibroblastic stroma

Allelotype analysis using microsatellite analysis, because of its simplicity and rapidity, represents the method of choice to detect chromosomal alteration in DNA from two matched cellular populations. While however this approach is being widely and successfully used to study cancer - including prostate - versus normal cells, technical and conceptual limitations impede its applicability to the study of fibroblasts. The major obstacle is due to the fact that in the tumor stromal fibroblasts are limited as compared to the cancer cells and bulk analysis of total DNA extracted from a tumor will predominantly show changes affecting cancer cells. Investigators, in order to overcome this limitation have applied micro-dissection in order to isolate fibroblasts from epithelial cells; however, unavoidably in this case they target the analysis to clonal fibroblastic populations that are close but not *within* the tumor. We propose to overcome this problem as follows: In previous experiments in



our laboratory studying the role of stromal p53 in tumorigenesis we have shown that in tumors growing in animals heterozygous for p53, stromal fibroblasts occasionally exhibited loss of heterozygosity (LOH) and reduction to hemizygosity for the mutant allele. The methodology used to show this alteration involved total DNA isolated from the xenografts that was subsequently subjected to LOH analysis by using mouse-specific primers for PCR. Based on this finding, and considering that by using species –specific primers the stromal component of a xenograft can be analyzed independently from the neoplastic, we propose to perform a detailed allelotype analysis of stromal cells. The analysis will involve xenografts of prostate cancer cell lines PC-3 and LNCaP that will be injected s.c. into SCID mice that will be wild type, p53 null or p21/waf1^{+/-}. p21-null hosts will not be included because according to our experience p21 deficiency in SCID background dramatically increases the mortality of the experimental animals. The reason that various genotypes of the hosts will be included, in respect to the status of p53 and p21, is because the rate of genomic instability will be increased in the fibroblasts facilitating the intended analysis.

In summary, 3 genotypes of SCID hosts will be used, wild type, p53 null or p21/waf1^{+/-}, each of them inoculated with both PC-3 and LNCaP cells (n=8 mice per genotype per cell line). Thus, 48 tumors will be generated (2 cell lines X 3 genotypes X 8 mice per group), and will be subjected to LOH analysis by using about 100 polymorphic microsatellite markers spanning all 19 mouse chromosomes. It has to be emphasized that it is of fundamental significance the selection of appropriate markers. Ongoing studies in our laboratory have and continue to identify such markers that are specific for mouse genome and do not amplify human DNA. Identification of such markers is a perquisite for our analysis because, as already mentioned above will permit the selective analysis of the mouse (stromal) DNA that is "contaminated" with human (neoplastic from the corresponding cell lines) DNA.

Exploration of the therapeutic potential of modulating prostatic stroma fibroblasts

As mentioned above we have identified that p21 in the stromal fibroblasts and KDELr3 and ERp29 endoplasmic reticulum –associated proteins in the cancer epithelial cells play an important regulatory role in the determination of the efficacy of the interaction between cancer cells and stromal fibroblasts. It is therefore conceivable that modulation of their expression to alter and importantly suppress, the kinetic profile of carcinogenesis. The current objective is to test this hypothesis in an *in vitro* system that is based on transwells and at which fibroblasts and cancer cells are being co-cultured. The analysis will involve PC-3 and LNCaP prostatic carcinoma epithelial. In this set of experiments we will modulate the expression of p21 in the stromal fibroblasts or KDELr3 and ERp29 in the cancer epithelial cells by using plasmid – mediated RNA-interference – based approaches and then assess if and how the proliferation and the morphology of the cancer cells is altered. The rate of cell proliferation will be performed by direct counting in association with trypan blue exclusion, while for morphological evaluation the morphology of the cancer cells will be assessed under both, growth in liquid and semisolid media. RNAi-based approaches are preferred



over pharmacological inhibition because the latter unavoidably will affect both cell populations (prostatic epithelial cells and stromal fibroblasts) and complicate our findings.

Our working hypothesis is that upon interaction of the cancer cells with the stromal fibroblasts the first elicit signals by mechanisms that involve the activation of ERp29 and KDELr3. The latter receive these signals and in turn respond by eliciting other signals that result in the promotion of neoplastic growth. p21 during this process in the fibroblasts initially is being stimulated playing a negative role and subsequently is being suppressed playing a positive role in cancer cell proliferation. Based on this hypothesis it is anticipated that in example, when ERp29 or KDELr3 are suppressed in the cancer cells, interaction with the stromal fibroblasts is defective and cancer cell proliferation is inhibited.

Diagnostic significance of stromal fibroblasts

We will evaluate the potential diagnostic and prognostic significance of p21/waf1 expression in stromal fibroblasts from prostatic carcinoma. The corresponding analysis will be performed in parallel by both immunochemistry and *in situ* hybridization to detect expression at both the protein and RNA level respectively. The analysis will involve a bank of about 150 matched cytololgical and histological specimens spanning all stages of prostate cancer development, from fibroadenomas (benign lesion) to prostatic intraepithelial neoplasia (PIN, presumably the earliest lesion underlining the malignant conversion of the mammary epithelium) to invasive adenocarcinomas of the prostate. Samples are already available in our laboratory and were obtained from the Laboratory of Pathology, Aretaieion University Hospital, Athens. This set of specimens will be subjected to staining for p21 and the positivity in the fibroblasts (stromal) will be scored. Subsequently, the results of p21 positivity in the fibroblasts will be analyzed in association with the type of breast cancer to assess if p21 expression (in the stromal fibroblasts) occurs and at which stage (early or late) during prostate cancer progression.

In addition, the epithelial component of these prostate cancer specimens will be subjected to staining for markers for cell proliferation such as proliferating cell nuclear antigen (PCNA), Ki-67, cyclin D1 and others, as well as markers for apoptosis such as various caspases and the TUNEL assay to address if the degree of p21 positivity in the stromal fibroblasts is related to the kinetic profile of the cancer cells. The latter, is considered to be in direct association with the aggressiveness of the disease. The aforementioned analysis will answer whether the relative levels of p21 expression in the stromal fibroblasts possesses a diagnostic and prognostic significance for prostate cancer.

Results, Conclusions and Future Perspectives

To study if any interference between cancer cells and stromal fibroblasts (fibs) exists we used primary stromal fibroblasts from mice and human prostate cancer cell line PC3. As p53/p21^{WAF1} pathway is frequently deregulated in stromal cells we used fibs differed in their p53 status. PC3 cancer cells were cultured in the presence of conditioned media from p53wt or p53-/- fibs and vice versa. The growth rate was evaluated by direct cell counting in association with trypan blue



exclusion. PC3 cancer cells cultured in the presence of conditioned media from p53wt fibs had dramatically decreased proliferation rate while those grown in the conditioned media from p53-/- fibs did not slow their proliferation rate; instead they had a slight increased proliferation rate. Whilst, both p53wt and p53-/- fibs decreased their proliferation in the presence of conditioned media from PC3 cancer cells. We therefore showed a paracrine communication between cancer cells and their microenvironment, with the conditioned media from p53 null fibs promoting the proliferation of PC3 cancer cells.

Additionally, *in vitro* wound healing assay was performed to study the motility and migration of cancer cells using scratch assay. Fibroblasts with p53 deficiency grown in conditioned media from PC3 cancer cells migrated 2 times faster than their control or p53wt counterparts, while PC3 cells grown in conditioned media from null fibs migrated slower than the control or the PC3 cells grown in the conditioned media from p53wt fibs. The stimulated PC3 cell migration reflects the degree of cellular motility which is associated with the metastatic potential of cancer cells.

To further investigate the paracrine stimulatory mechanism and if this affects viability of PC3 cancer cells bearing stem cell-like properties, we searched for CD44(hi)/CD24(low) marker profile by flow cytometry in PC3 cells co-cultured with wild-type or p53 deficient fibs. Flow cytometry revealed that PC3 cells lost their cancer stem cell-like profile when co-cultured with wild-type p53 fibs and restored the CD44(hi)/CD24(low) profile when co-cultured with p53 deficient fibs.

Our findings suggest that deregulated expression and mutations in p53 in stromal fibs are linked to tumor growth, affecting the proliferation rate as well as migration and stem-cell content. Hence, modulation of p53/p21^{WAF1} activity in stromal fibs virtues further investigation. To do so we will silence p21^{WAF1} in p53-/- stromal fibs using siRNA method to investigate the molecular pathway following p53 deregulation. The conditioned media will be applied to PC3 cancer cells and proliferation, migration and cancer stem cell marker will be assessed to study the impact of p21^{WAF1} silenced p53-/- stromal fibs on PC3 cells.

Increased migration will be studied through smooth muscle actin (SMA). Western blot analysis will reveal if SMA increased along with increased migration of PC3 cancer cells cultured in conditioned media from p53-/- stromal fibroblasts. RNA silencing of SMA will demonstrate if SMA is necessary for migration of PC3 cells.

List of Publications Resulting from the Grant

Karamouzis MV, et al. "Essential role of p53 of stromal fibroblasts (fibs) in the reciprocal interaction between PC3 prostate cancer cell and stromal fibs", ASCO 2011, e21009



Selection of Courses & Workshops Attended During the Fellowship

ASCO 2011 ESMO 2011 ASCO 2012 ESMO 2012

600

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