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13. ABSTRACT (Maximum 200 Words)
We hypothesize that genes that are differentially expressed as a result of the decreased IGF-I receptor gene expression seen in metastatic prostate cancer contribute to prostate cancer progression, and include metasasis-regulating genen sthat could constitute valuable diagnostic markers or therapeutic targets. We initially proposed three specific aims: 1)Identification of differentially expressed genes in isogenic metastatic vs. non-metastatic prostate epithelial cells; 2)Identification of proteins that are differentially secreted in these cell lines, and 3) Assessment of he differential expression of these genes and proteins in laser-mircrodissected samples. We have used microarray gene profiling to characterize differentially expressed genes and have used SELDI-TOF mass spectrometry to identify proteins that are differentially secreted into conditioned media. We have additionally initiated a 3 dimensional culture system to grow prostate cells in a microgravity environment that more accurately replicates in vivo cell organization and phenotype. We have discovered that elevated IGF-I receptor expression controls survival in adult human male serum, which may explain the relationship between IGF action and metastasis. We propose to take advantage of these new findings by investing the molecular mechanisms underlying this effect and the cell-surface molecules expressed in metastatic and non-metastatic cells responsible for the differential sensitivity to serum.

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Introduction: The insulin-like growth factor (IGF) signaling system plays an important role in the initation and progression of prostate cancer. High circulating levels of IGF-I are associated with an increased risk of developing prostate cancer, and constitutive expression of IGF-I in the prostate epithelium of transgenic mice results in neoplasia. The actions of IGF-I are mediated through activation of the IGF-I receptor, a transmembrane tyrosine kinase that is highly expressed in normal prostate epithelium and immortalized prostate epithelial cell lines. A significant *decrease* in IGF-I receptor expression is seen in metastatic prostate cancer cell lines, human metastases, and metastatic lesions from transgenic mouse models of prostate cancer. Retroviral re-expression of the IGF-I receptor in metastatic prostate cancer cells reduces their tumorigenicity and metastatic potential. These data suggest that, while IGF-I action may contribute to the initiation of prostate cancer, a subsequent loss of IGF responsiveness secondary to reduced IGF-I receptor expression is necessary for progression to advanced disease. This requirement for decreased IGF-I responsiveness may reflect the ability of the activated IGF-I receptor to exert differentiative as well as proliferative effects. Our hypothesis is that genes that are differentially expressed as a result of the decreased IGF-I receptor gene expression seen in metastatic prostate cancer contribute to prostate cancer progression. Specifically, IGF-I receptor target genes may include metastasis-promoting or suppressing genes that could constitute valuable diagnostic markers or therapeutic targets. To evaluate this hypothesis, we proposed three specific aims: 1) Identification of a select subset of genes that are differentially regulated in otherwise isogenic metastatic vs. non-metastatic prostate epithelial cells that differ solely in the level of expression of the IGF-I receptor; 2) Identification of proteins that are differentially secreted in the cell lines used in aim 1, and 3) Assessment of the differential expression of these genes and gene products in laser-microdissected samples from normal prostate, adenocarcinoma, and metastatic lesions. The studies of specific aim 1 employ microarray gene profiling of metastatic prostate epithelial cells and their non-metastatic counterparts that are re-expressing the IGF-I receptor from a retroviral construct. The studies of aim 2 employ surface-enhanced laser desorption-ionization/time-of-flight (SELDI-TOF) mass spectroscopy to identify proteins that are differentially present in the conditioned media of the two cell types utilized in aim 1. Subsequent tandem mass spectroscopy analyses will be performed to generate proteolylic cleavage patterns or peptide sequences for gene identification. The studies of specific aim 3 will evaluate the expression of IGF-I receptor target genes in a series of human clinical samples using quantitative real-time RT-PCR analyses. The proposed studies address a critical aspect of prostate cancer, i.e., factors that contribute to the development of advanced disease. The studies underway employ an innovative, integrated, gene and protein profiling approach in a novel, carefully defined and highly controlled model system to identify genes and gene products that regulate metastasis. Most recently, we have discovered that IGF-I receptor expression controls survival in human adult male serum, a novel finding that suggests that IGF action may regulate the initial step in the metastatic process, i.e., intravasation from the primary tumor. These data are of major importance in light of recent studies highlighting the critical role of intravasation in cancer progression (1-3).

Body: The approved statement of work included three tasks that were to be initiated in the first 24 months of funding. These tasks were in support of aims 1 and 2 of the proposed project. These will be discussed in turn.

Task 1: Completion of microarray analysis of genes differentially expressed in LISN and LNLG cells that express different levels of IGF-I receptor and which are, respectively, non-metastatic and metastatic in nude mouse xenografts. We have completed this analysis using three independent RNA preparations from each cell line grown in defined medium with 5% FBS and have analyzed each sample using triplicate arrays that each contain >12,000 sequence-verified, non-redundant human cDNA clones. Data have been analyzed by accepted means of normalization, statistical verification and false-discovery rate analyses. These data demonstrate that there are specific genes that are constantly differentially expressed in LISN and LNLG cells. We have recently acquired a rotary cell culture system for three-dimensional (3-D) cultures of cells in a microgravity environment. This NASA-designed apparatus had been used to propagate cells under conditions that alter them to form 3-D structures that may be more similar to the in vivo situation (1,2). Indeed, a recent report has shown that tumor cell lines grown under microgravity conditions display a phenotype that is much more similar to that of clinical samples in situ than cells grown in monolayer cultures (6). This system has been used in several

previous studies to grow prostate cells in particular (7-10). We have now employed this novel culture system to repeat the array analysis that constituted task 1, since we feel that the data obtained will be significantly more relevant than that obtained with standard monolayer culture. These gene-profiling studies are currently being completed and will be summarized in the final report.

Task 2: Completion of preliminary SELDI-TOF analysis of conditioned media from M12-LISN and M12-LNL6 cultures. Cells were grown in defined media supplemented with 5% FBS under either standard tissue culture conditions or in a 3-dimensional high-aspect rotating wall vessel system that more closely mimics in vivo conditions. Conditioned medium (CM) was collected 72 hours after culture initiation, acetone-precipitated, ethanol-washed, dissolved in 6M urea, and, for some experiments, size-fractionated using Centricon YM-50 columns to remove serum albumin. CM extracts (1-μg aliquots) were then applied to either NP20 normal-phase protein array chips or WCX-2 cation-exchange protein array chips and analyzed at 220, 250 and 270 laser intensity on a Ciphergen PBS-II platform. SELDI-TOF profiling revealed a discrete set of proteins in the 4-25 kDa range that were differentially present in the CM of the LNL6 and LISN lines grown under 2-dimensional versus 3-dimensional conditions, as well as proteins that were differentially present in CM of LNL6 and LISN cells under either culture condition. Some of these proteins were differentially expressed between the different lines under both conditions.

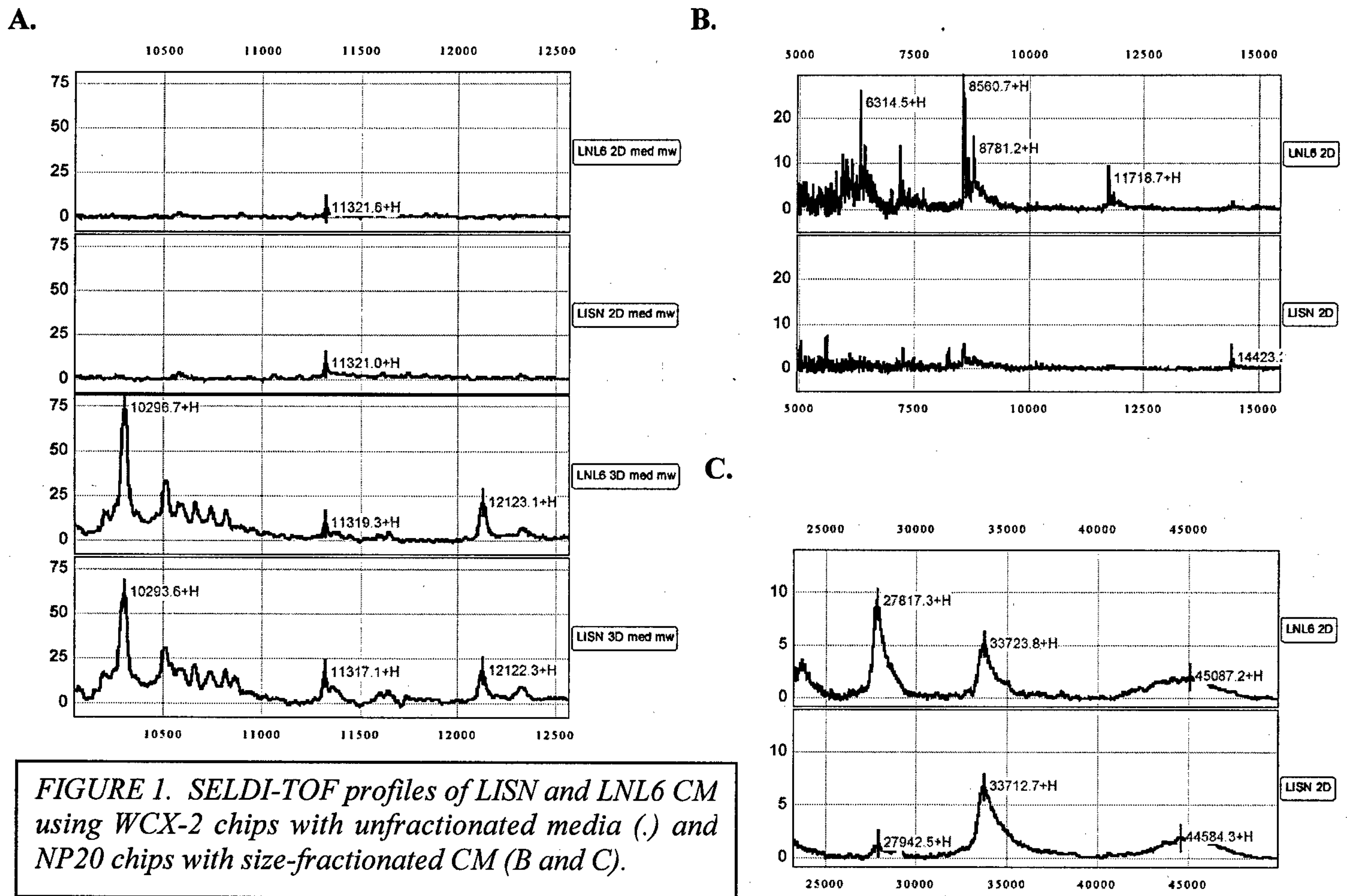

FIGURE 1. SELDI-TOF profiles of LISN and LNL6 CM using WCX-2 chips with unfractionated media () and NP20 chips with size-fractionated CM (B and C).

As shown in Figure 1A, the secreted protein profile was more complex in cells grown under 3-D conditions vs. standard 2-D conditions (compare lower two profiles to upper profiles). The profiles obtained with the NP20 chips did not reveal any differences between metastatic and non-metastatic cells, however. When fractionated CM extracts were analysed with WCX-2 chips in the low (B.) and higher (C.) molecular weight ranges, it was found that metastatic LNL6 cells secreted 6, 8, 11, and 27-kDa proteins that were not seen in LISN CM, while non-metastatic LISN cells secreted a 14-kDa species that was not detected in LNL6 CM. Thus, changes in IGF-

I receptor expression of the degree characteristic of metastatic versus non-metastatic prostate cancer cells are associated with alterations in the secreted protein profile. We are currently identifying these differentially expressed proteins by preparative gel electrophoresis followed by tandem mass spectrometry. We are also initiating an effort to identify differentially expressed cell-surface proteins by isolating limiting trypsin digest fractions of LNL6 and LISN cells prior to SELDI-TOF characterization.

Task 3: Generation of probes for Northern and/or RPA analysis and verification of differential gene expression in M12-LISN and M12-LNL6 cells. We have begun to design primers of generation of cDNA probes for some of the robustly differentially expressed genes identified in the array studies of task 1, but will first determine which of these are differentially expressed in these cell lines grown in 3-D culture before proceeding further.

Tasks 4-8 concerned the evaluation of differentially expressed genes and proteins in clinical samples. In light of our recent exciting findings regarding the control of serum survival by IGF-I receptor expression level (described below), we feel that it would be more informative and of direct mechanistic relevance to assess the molecular basis for this phenomenon.

We initially hypothesized that cells with decreased IGF-I receptor expression would be more metastatic because they would potentially be more resistant to the possibly differentiative effects of IGF-I encountered at sites such as bone. This hypothesis did not, however, satisfactorily explain the observation that restoration of IGF-I receptor expression reverses both metastatic capacity and tumorigenecity in nude mouse xenografts. We then considered the possibility that cells that maintained high levels of IGF-I receptor expression would be subject to programmed cell death triggered by the normally anti-apoptotic IGF-I receptor when they encountered a high-trophic support environment such as 100% serum. This notion was supported by the experiment shown in Figure 2 below, which shows the survival (measured as cells attached to tissue culture dishes) of LNL6 and LISN cells transferred from media containing 5% FBS to 100% human adult male serum.

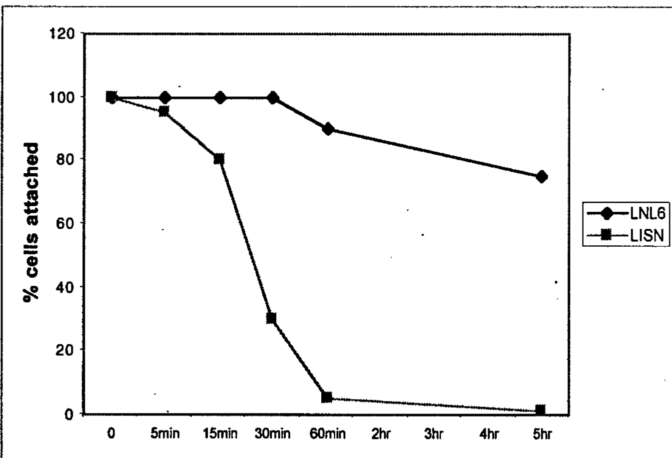

Figure 2. Percentage of LISN and LNL6 cells attached to tissue culture plates following transfer to 100% adult human male serum.

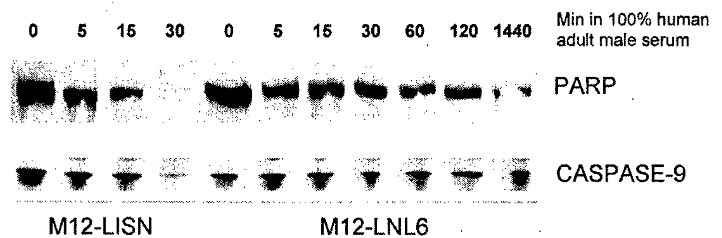

Figure 3. Western immunoblot analyses of PARP and caspase-9 cleavage in LISN and LNL6 cells following transfer to 100% adult human male serum. Attached cells were harvested at the indicated times and lysed in SDS-PAGE loading buffer prior to gel electrophoresis and transfer to nitrocellulose membranes. Signal corresponding to primary antibody binding was visualized with enhanced chemiluminescence.

As shown in Figure 3, detachment of non-metastatic LISN cells was preceded by PARP and caspase-9 cleavage, which was not seen in metastatic (and serum-resistant) LNL6 cells. These data suggest that serum exposure elicited programmed cell death, although we cannot yet conclude whether this represents classical apoptosis or paraptosis. We are currently assessing the signaling pathways that regulate IGF-I receptor-mediated serum sensitivity, as well as the behavior of LNL6 and LISN cells exposed to 100% dault human male serum under 3-D culture conditions.

Key research accomplishments:

- Demonstration of differential gene expression profiles in M12-LISN and M12-LNL6 cells expressing different levels of IGF-I receptor.

- Identification of specific molecular weight species that are differentially secreted by metastatic and non-metastatic prostate cancer cells under 2-D and 3-D culture conditions.

- Discovery of differential survival of cells with different IGF-I receptor expression levels in 100% adult human male serum, but not in 100% fetal bovine serum.

- Initial characterization of the signaling and programmed cell death pathways involved in sensitivity of IGF-I receptor-expressing cells to human adult male serum.

Reportable outcomes: (supported in part by this award)

- Abstract describing SELDI-TOF analyses of LISN and LNL6 cells in 2-D and 3-D culture to be presented at 86th Annual Meeting of the Endocrine Society in New Orleans, LA, June, 2004. Denley, A., Carroll, J.M., Nagalla, S.R., and Roberts, C.T., Jr., Proteomic analysis of IGF-regulated proteins in prostate cancer cells. (See appendix).

- Research article describing interactions between IGF and androgen receptor signaling in LISN and LNL6 cells and derivatives accepted for publication. Plymate, S.R., Tennant, M.K., Culp, S.H., Woodke, L., Marcelli, M., Colman, I., Nelson, P.S., Carroll, J.M., Roberts, C.T., Jr., and Ware, J.L. Androgen receptor (AR) expression in AR-negative prostate cancer cells results in differential effects of DHT and IGF on proliferation and AR activity between localized and metastatic tumors. Prostate, in press (2004).

- Research article describing effect of saw palmetto extract on IGF signaling in P69 prostate cell line parent of LISN-LNL6 system accepted for publication. Wadsworth, T.L., Carroll, J.M., Roberts C.T., Jr., and Roselli, C.E. Saw palmetto extract suppresses IGF-I signaling and induces SAPK/JNK phosphorylation in human prostate epithelial cells. Endocrinology, in press (2004).

- Idea Development Award application on androgen receptor regulation of IGF-I receptor expression submitted to FY04 PCRP, February, 2004.

Conclusions: We have shown that differences in IGF-I receptor gene expression that are sufficient to alter metstatic capacity are sufficient to alter gene expression and secreted protein expression profiles in monolayer cell culture. There is an increasing appreciation that findings made in monolayer cell culture may not accurately reflect the molecular situation in vivo, as compared to what can be achieved with 3-D culture approaches (4, 11-13). Perhaps more importantly, we have discovered a direct correlation between IGF-I receptor expression level and survival in serum, a difference that may reflect intrinsic differences in the ability of cells to successfully intravasate from the primary tumor into the circulation, now considered to be the critical event in tumor progression to metastasis. These findings may reflect a direct relationship between IGF-I receptor action and metastasis; thus, an understanding of the molecular mechanisms responsible may elucidate novel aspects of prostate cancer progression and identify previously unappreciated therapeutic targets.

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Appendices: See attached.

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Title: Proteomic Analysis of IGF-Regulated Secreted Proteins in Prostate Cancer Cells

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The IGF system regulates normal growth and development as well as playing an important role in tumorigenesis. Studies of human prostate cancer cell lines, human clinical specimens, and the TRAMP model of prostate cancer have described a decrease in IGF-I receptor (IGF-IR) expression in metastatic prostate cancer. The functional importance of the down-regulation of IGF-IR expression in prostate cancer progression is supported by the inhibition of metastatic capacity that follows restoration of IGF-IR expression in metastatic prostate cancer cells. Using a well-characterized cell culture model comprised of a tumorigenic and metastatic M12 prostate cancer cell line infected with a control retrovirus (LNL6) and a poorly tumorigenic and metastatic derivative (LISN) in which IGF-IR expression has been restored with a retroviral expression construct, we have employed surface-enhanced laser desorption-ionization/time-of-flight (SELDI-TOF) mass spectrometry to profile secreted proteins that are differentially expressed in these cell lines that differ solely in their level of IGF-IR expression. Cells were grown in defined media supplemented with 5% FBS under either standard tissue culture conditions or in a 3-dimensional high-aspect rotating wall vessel system that more closely mimics in vivo conditions. Conditioned medium (CM) was collected 72 hours after culture initiation, acetone-precipitated, ethanol-washed, dissolved in 6 M urea, and

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References:
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