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THE EFFECTS OF LEPTIN TREATMENT ON STAT/AKT/ERK SIGNALING PATHWAYS IN CRL2091 FIBROBLAST AND HT1080 FIBROSARCOMA CELLS

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ABSTRACT

The hormone leptin has been known to play an integral role in the development of obesity. More recent research has led to the discovery of the association between circulating leptin levels and the development and progression of carcinomas in various organ sites. It has been demonstrated that binding of leptin to its receptor on carcinoma cells leads to phosphorylation and activation of signaling proteins enhancing cellular proliferation, survival, migration, and invasion. However, the effect of leptin on normal and cancer cells of mesenchymal origin has been largely unknown. We hypothesized that leptin effects mesenchymal cells by activating signal transduction pathways and conducted experiments with a view to elucidating the effects of leptin on the phosphorylation status of STAT3, ERK1/2, and AKT1/2/3 in fibroblast (CRL2091) and fibrosarcoma (HT1080) cells. The results of the study confirm the hypothesis and start to provide mechanistic insights on the observed leptin-induced increase[1] in proliferation, migration, and invasion of leptin treated CRL2091 and HT1080 cells.

[1] Observations from other experiments conducted at the Kanjilal laboratory

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Introduction

Leptin is a small molecular weight hormone that is secreted primarily by adipose tissues and circulating leptin levels in the blood bear a direct relationship with total adipose tissue mass (Masuzaki 1996). Leptin was initially discovered in a strain of spontaneously obese mice that lacked the protein due to mutations and was widely known as the "obesity hormone" (Maffei 1995, Alexe 2006, Ingalls 1950). The protein was subsequently discovered in humans where obesity is linked to leptin resistance, which results in hyperleptinemia (Alexe 2006). Aside from adipocytes, this 16-kDA protein is also found in gastric epithelial cells as well as placental cells during pregnancy, and to some extent various other tissues including testes, ovaries, mammary glands, hair follicles, bone and muscle tissues (Stallmeyer 2001, Li 2012, Smith-Kirwin 1998, Wang 1998, Hoggard 1997).

For decades after the discovery of leptin, research in numerous laboratories centered on the hormonal function of the protein in regulating lipid and carbohydrate metabolism and energy balance through its action via receptors in the hypothalamus (Hamann 1996, Alexe 2006). However, in recent years many other functions of leptin have emerged and the hormone has been found to play a key role in a broad variety of normal physiologic processes including angiogenesis, hematopoiesis, bone development, immunity, and reproduction as well as in the development of pathologic conditions such as diabetes, cardiovascular disease, and cancer (Huang 2000).

The link between leptin and chronic diseases such as cancer has serious implications for our society in which obesity levels have reached epidemic proportions (Klein 2002). Obese populations characteristically have elevated insulin and leptin levels and are documented to have a greater likelihood of developing cancers, as well as other inflammation driven chronic health conditions (Alexe 2006, Klein 2002). The relationship between leptin and insulin plays a role in

carcinogenesis in obese individuals as insulin has been found to increase leptin secretion leading to higher serum concentrations of the growth-promoting cytokine (Askari 2000). Leptin levels are increased in several cancerous tissue types including those of the breast, colon, prostate, pancreas, and endometrium (Ishikawa 2004, Wu 2002, Koda 2007). One study found that leptin was expressed in higher concentrations in 92% of breast cancer cells relative to normal breast tissue and evidence is accumulating on the direct relationship between expression of this hormone and tumor grade (Saxena 2008, Ishikawa 2004, Garofalo 2006).

Leptin exerts its influence on cancer development by inducing the expression of a number of factors. Exposure of human eosinophils to leptin has been demonstrated to result in enhanced expression of certain adhesion molecules on cell surfaces, such as ICAM-1 and CD18, and release of inflammatory cytokines such as IL-1B, IL-6, and IL-8 (Wong 2007). It is also known to increase the expression of MMPs (matrix metalloproteinases, or tissue modeling enzymes) 1, 2, and 9, TIMPs (tissue inhibitors of metalloproteinases), and VEGF (vascular endothelial growth factor), which help in vascularization, angiogenesis, and cell migration (Park 2001). Moreover, exposure to leptin can induce cellular growth genes such as c-fos, c-jun, junB, and egr-1 as well as anti-apoptotic genes such as bcl-2 (Bouloumie 1998, Artwohl 2002, Sweeney 2002). The emerging theme from the numerous studies that have examined the role of leptin in the development of tumors, including carcinoma of the breast, colorectal, prostate, ovarian, and lungs, is that leptin acts as a cytokine and stimulates cell proliferation, anchorage-independent growth, migration, and invasion in epithelial and hematopoietic cells (Garofalo 2006, Wong 2007, Alexe 2006).

Interest on the role of leptin in the development and progression of many types of cancer has prompted the investigation of leptin-mediated signaling (Alexe 2006). Leptin functions through a transmembrane receptor at the cell surface that is structurally similar to the class I family of cytokine receptors (Tartaglia 1995). The leptin receptor has a number of isoforms, the

longest of which contains domains that trigger signal transduction upon activation (Ghilardi 1996). Aside from the hypothalamus, where leptin was initially found to exert its activities related to maintenance of body mass, the long-form receptor is expressed in many tissue and cell types (Garofalo 2006). Binding of leptin to the long-form receptor leads to a cascade of phosphorylation that causes activation of STAT3, AKT, and ERK 1 and 2[2] (Saxena 2007, Wong 2007). These activated signal transducers alter the expression levels of a large number of genes resulting in dose/time-dependent changes in cell proliferation, survival, migration, and invasion (Saxena 2007).

Hypothesis and Specific Aims

There is paucity of information on the effect of leptin on normal cells and cancers of mesenchymal origin. The experiments described herein were undertaken with a view to elucidating the effects of the cytokine on the activation status of STAT3, ERK1/2, and AKT in mesenchymal cells. It was hypothesized that leptin would exert its effects on the mesenchymal cells by increasing the phosphorylation status of one or more of these proteins, thereby activating signal transduction, and leading to phenotypic changes such as increased proliferation, migration, and invasion.

Specifically, it was proposed to treat normal fibroblast cells (CRL2091) and malignant fibrosarcoma (HT1080) cells with 200 ug/mL of leptin for different time intervals ranging from 0 (control) to 8h in absence of other serum growth factors and to use the method of Western blot analysis to measure the activation status of the following key components of signaling pathways:

[2] The shorter forms of the leptin receptor have truncated intracellular domains and limited participation in signaling pathways. However, these short forms of the leptin receptor may play a role in leptin transport and regulation of ligand-interaction with the long form of the receptor (Hileman 2000, Fruhbeck 2006).

I) STAT3: Level of phosphorylation at Tyr705 and Ser727 residues as compared to the total amount of STAT3

II) ERK1/2/3: Level of phosphorylation at Thr202/Tyr204 residues as compared to the total amount of ERK

III) AKT1/2: Level of phosphorylation at Ser473 as compared to the total amount of the AKT

Significance

This study examines for the first time if fibroblast cells respond to leptin either in their normal or malignant condition. Hence, the results of the study have important implications on the link between obesity and the development and progression of fibrosarcoma. The information has potential to help in promoting a disease prevention lifestyle as well as aid in the management of fibrosarcoma, which may be aggravated, by elevated leptin levels in obese or diabetic patients. Moreover, knowledge of the effect of leptin on signaling pathways in normal fibroblasts has major implications with regards to the development of a number of other serious chronic conditions, such as arthritis and cardiovascular disease, as well as in wound healing. Thus, ongoing work at the laboratory is focused on unraveling key alterations caused by exposure of normal cells to the elevated levels of leptin found in obese individuals.

Methods

Cell Culture

Fibroblast (CRL2091) and epithelial fibrosarcoma (HT1080) cells were cultured in complete medium (consisting of DMEM supplemented with 10%FBS, 100U/mL penicillin, 100mg/mL streptomycin, and 250 mg/mL amphotericin B) at 37°C under 5% CO_2. The cells were grown until 60% confluent and serum-starved for 16h before being treated with 200ng/mL leptin for various time intervals from 15m to 8h. Corresponding control cells were processed in an identical manner except that they were not treated with leptin.

Protein Extraction

Tissue culture plates containing 80-90% confluent cultures were washed two times in PBS after which the cells were lysed using the mammalian protein extraction reagent (M-PER) supplemented with 1mM NaF, 1mM activated sodium orthovanadate, and Complete Mini Protease Inhibitor Cocktail tablets at the manufacturer's recommended amount of one tablet per 10 mL of the lysis buffer. The protein content of the extracts were determined using the Bradford protein assay reagent and a standard curve prepared by two-fold serial dilution starting from 1.5ug/ml and ending with 0.023438 ug/mL of BSA. The protein concentrations of the extracts were extrapolated from the linear portion of the standard curve.

Western blotting (Immunoblotting)

Aliquots of the cell lysates for Leptin-treated HT1080 and CRL2091 cells and their corresponding untreated controls containing 25ug of protein were mixed with equal volumes of Laemmli sample loading buffer consisting of 95% Laemmli sample buffer and 5% beta-mercaptoethanol, heated at 95°C for 10 minutes in order to denature the proteins, and

electrophoresed through 8% or 10% SDS-PAGE gels. The proteins contained in the gels were then transferred for 1.5h onto PVDF membranes using the Western blot technique (Gallagher SR Current Protocols in Cell Biology (2008) 6.0.1-6.2.20). Membranes containing the transferred proteins were then blocked with 5% skim milk in PBS for 30 m, cut horizontally to represent different molecular weight ranges, and incubated overnight in PBS containing 5% skim milk and a primary antibody (pSTAT3, mol. wt. 86kDa and 91kDa; pERK1/2, mol. wt. 42 and 44kDa; pAKT1/2/3, mol. wt. 56 and 60kDa; or GAPDH, mol. wt. 36kDa). On the second day, membranes were washed with PBS supplemented with 0.05% Tween, and incubated with the secondary antibody (conjugated to horseradish peroxidase) in a solution of 5% skim milk in PBS. The membranes were then treated with enhanced chemiluminescence substrate of appropriate sensitivity and exposed to an x-ray film, which was developed in a dark room. The membranes were then stripped with stripping buffer and reprobed for total STAT3, ERK, and AKT levels using a similar technique. Time course experiments were repeated at least two times for each target analyzed.

Data analysis

The developed films were scanned and analyzed with Image J densitometry software and Excel software was used to calculate the standard deviation of the band intensities in the scanned images. Band intensities for phosphorylated STAT, AKT, and ERK were each normalized against total levels of the proteins, and total levels were compared to levels of GAPDH, which served as a loading control. Fold change in intensity of the bands representing each of the proteins was plotted against time of treatment with leptin.

Results

Western Blotting was used in order to investigate if exposure to leptin activates signal transduction pathways in fibroblast (CRL2091) or fibrosarcoma (HT1080) cells by measuring the levels of total and phosphorylated STAT3, ERK1/2, and AKT1/2/3. In the HT1080 cell line, phosphorylation at the tyrosine residue at position 705 of STAT3 (pSTAT (Tyr 705)) was rapidly doubled within 15m of treatment with leptin (Fig 1 A & B). Phosphorylation of STAT3 at the site continued to increase to > 2.5 fold of the corresponding values for untreated control until 1h, whereupon the activation level remained relatively stable throughout the 8h time interval monitored (Fig 1 A & B). The response of CRL2091 fibroblast cells was initially more pronounced reaching 3.5 fold activation after 15m and 5 fold activation in 1h of leptin exposure but was partly attenuated to ~ 4 fold from the starting level between 1-4h (Fig 1 C & D). Expression decreased further from peak values over time, but was still 2.3-fold higher as compared to the control untreated cells at 8h (Fig 1 C & D). The total STAT3 levels remained level during the course of the leptin treatment.

Phosphorylation Status of STAT

In the HT1080 cell line, pSTAT (Ser 727) was not substantially changed throughout treatment with leptin (Fig 2 A & B). In the CRL2091 cell line, there was an initial increase in pSTAT (Ser 727) reaching 1.7-fold at 30m (Fig 2 C & D). Subsequently the phosphorylation levels remained largely constant, appearing to be 1.5 fold elevated relative to control at the 8h time point.

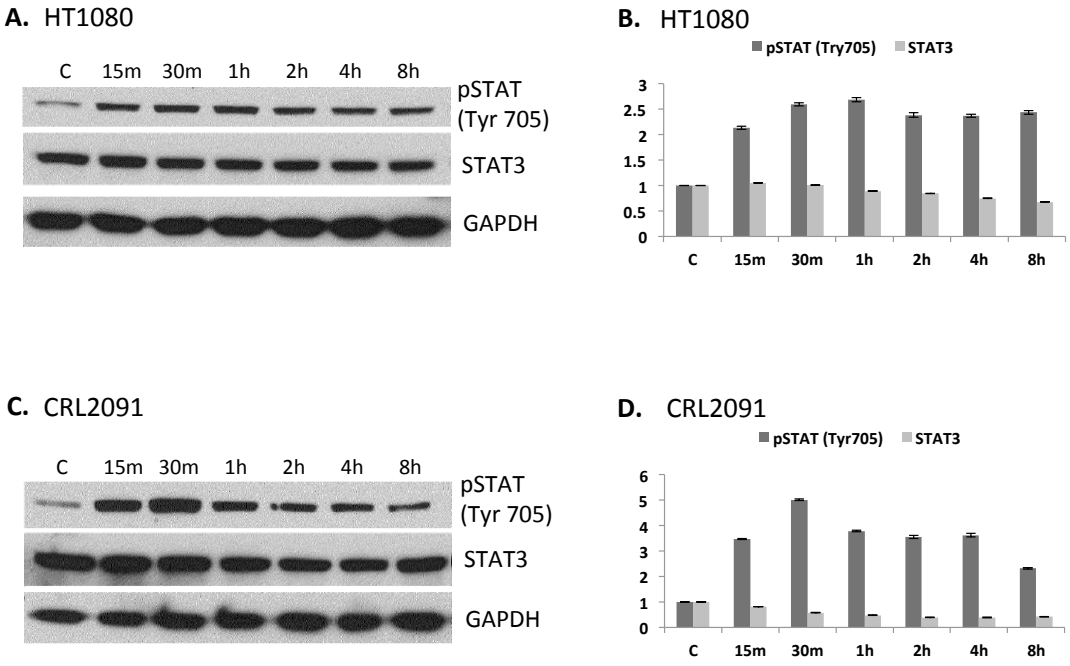

Figure 1. Effects of leptin on phosphorylation of STAT (Tyr 705) and total STAT3 levels in fibrosarcoma (HT1080) and fibroblast (CRL2091) cells. Cells were treated with 200ug/ml leptin for 0m (Control), 15m, 30m, 1h, 2h, 4h, and 8h. Figure 1A: Results of Western blot analysis of pSTAT (Tyr 705), STAT3, and GAPDH (used as loading control) for the HT1080 cell line. Figure 1B: Levels of phosphorylated STAT (Tyr 705) as compared to STAT3 and STAT3 levels as compared to GAPDH in HT1080 cells. Figure 1 C: Results of Western blot analysis of pSTAT (Tyr 705), STAT3, and GAPDH for the CRL2091 cell line. Figure 1 D: Levels of phosphorylated STAT (Tyr 705) as compared to STAT3 and STAT3 levels as compared to GAPDH in CRL2091 cells.

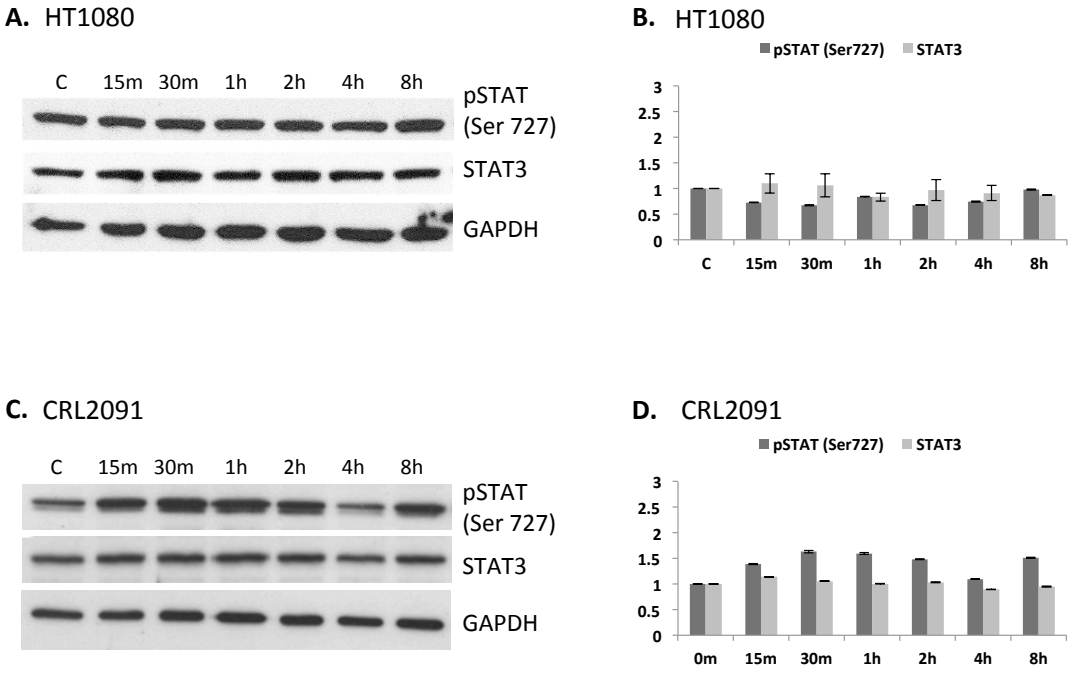

Figure 2. Effects of leptin on phosphorylation status at STAT (Ser 727) as compared to total STAT3 levels in HT1080 fibrosarcoma and CRL2091 fibroblast cells. Cells were treated with 200ug/ml leptin for 0m (Control), 15m, 30m, 1h, 2h, 4h, and 8h after which the cells were lysed and protein extracts subjected to Western analysis. Figure 2A: Western blot of pSTAT (Ser 727), STAT3, and GAPDH (used as loading control) for the HT1080 cell line. Figure 2B: Levels of phosphorylated STAT (Ser 727) as compared to STAT3 and STAT3 levels as compared to GAPDH in HT1080 cells. Figure 2 C: Results of Western blot analysis of pSTAT (Ser 727), STAT3, and GAPDH for the CRL2091 cell line. Figure 2 D: Levels of phosphorylated STAT (Ser 727) as compared to STAT3 and STAT3 levels as compared to GAPDH in CRL2091 cells.

Phosphorylation Status of ERK

The CRL2091 fibroblast cells also responded with a sharp 10 fold increase in ERK activation that peaked at 30 min after treatment and was mostly attenuated to 2.8-fold relative to control by 8h (Fig 3 C & D). HT1080 cells showed a small increase of 1.5 fold at 15m, followed with attenuation below starting levels at 30m. Thereupon the pERK level continued to decrease until it was only a third as much as the control at 8h (Fig 3 A & B). Total ERK remained similar to control levels in both HT1080 and CRL2091 cells throughout the 8h period of leptin treatment (Fig 3 A-D).

Phosphorylation Status of AKT

Neither CRL2091 fibroblast nor HT1080 fibrosarcoma showed a major response in terms of phosphorylation of AKT within the 8h time span of the experiment (Fig 4).

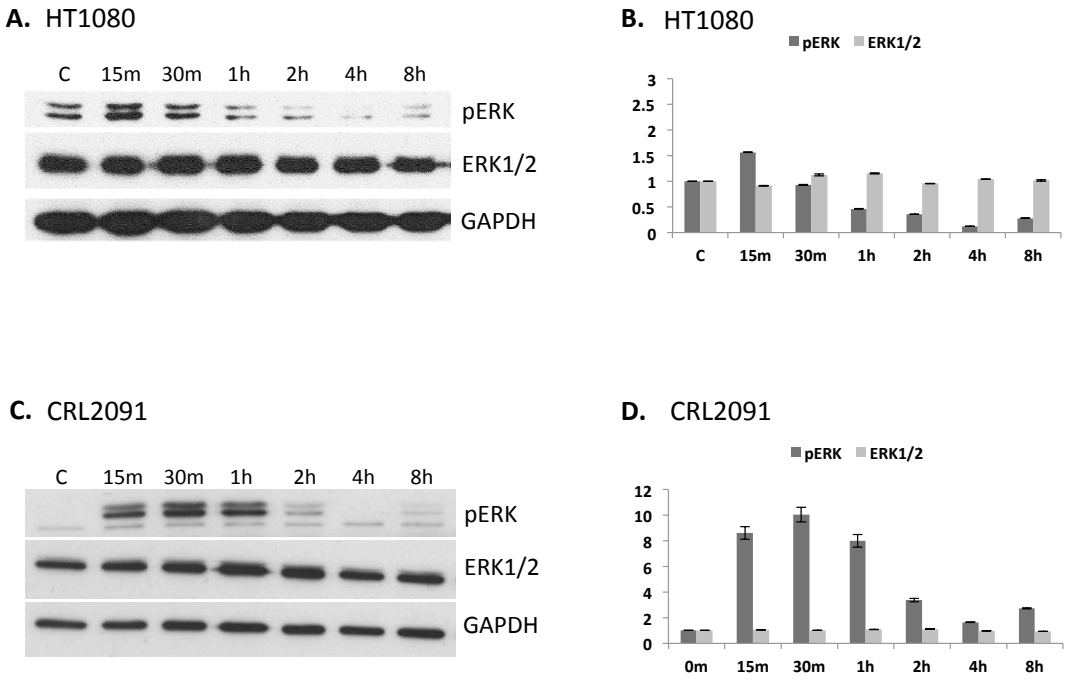

Figure 3. Effects of leptin on phosphorylated ERK and total ERK (ERK1/2) levels in HT1080 fibrosarcoma and CRL2091 fibroblast cells. Figure 3A: Western blot analysis of pERK, ERK1/2, and GAPDH (used as loading control) for the HT1080 cell line. Figure 3B: Levels of phosphorylated ERK as compared to total ERK1/2 and ERK1/2 compared to GAPDH in HT1080 cells. Figure 3C: Western blot analysis of pERK, ERK1/2, and GAPDH for the CRL2091 cell line. Figure 3D: Levels of phosphorylated ERK as compared to total ERK1/2 and ERK1/2 compared to GAPDH in CRL2091 cells.

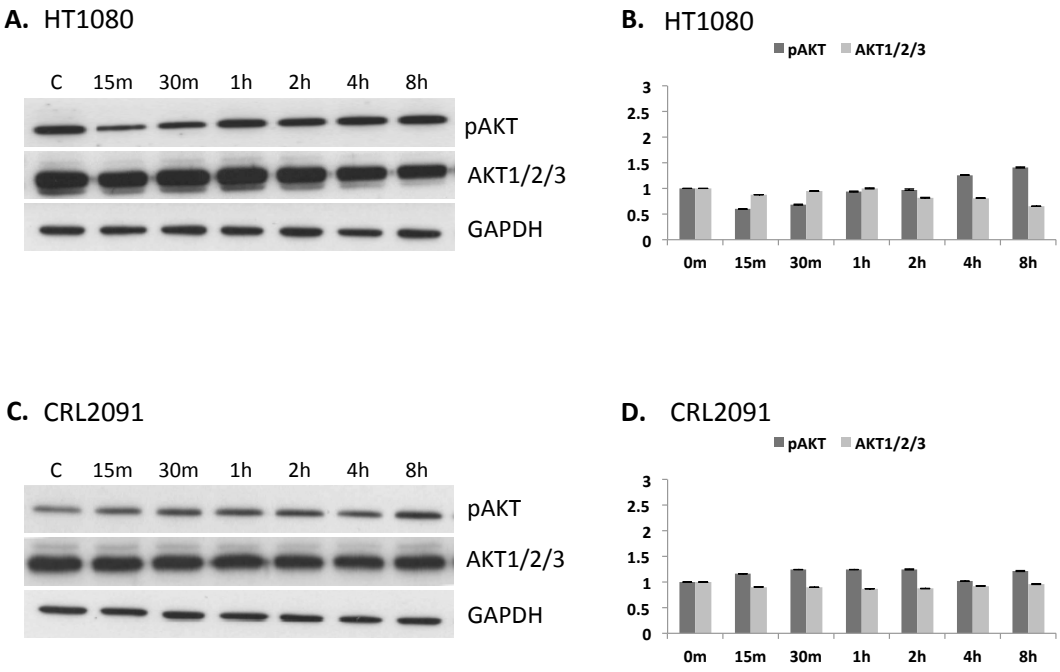

Figure 4. Effects of leptin on phosphorylation of AKT and total AKT1/2/3 levels in HT1080 fibrosarcoma and CRL2091 fibroblast cells. Figure 4A: Western blot of pAKT, total AKT1/2/3, and GAPDH (used as loading control) for the HT1080 cell line. Figure 4B: Phosphorylation status of AKT and total AKT1/2/3 levels. Figure 4C: Westerns for pAKT, total AKT1/2/3, and GAPDH for the CRL2091 cell line. Figure 4D: Phosphorylated AKT and total ATK1/2/3 levels in CRL2091 cells.

Discussion

Leptin plays an important role in the signaling pathways that lead to the development and progression of many types of cancer. The small molecular weight cytokine exerts its tumorigenic effects on these tumors by increasing a variety of cellular functions such as cell proliferation, survival, migration, and invasion via phosphorylation in STAT3, ERK, and AKT signaling pathways (Saxena 2007).

STAT3, a signal transducer and activator of transcription, is activated by cytokines and interferons via activation of JAK (Janus Kinase). When phosphorylated at the tyrosine residue at position 705, STAT3 forms a dimer and is transported to the nucleus, where it binds DNA and induces cellular transcription (Hazan-Halevy 2010). Phosphorylation at the serine residue at position 727 enhances the function of STAT3 as a transcription factor, however phosphorylation at either site has been demonstrated to potentiate cancer cell growth (Lai 2003). STAT3 is constitutively phosphorylated at Ser 727 in chronic lymphocytic leukemia cells and at Tyr705 in several forms of solid tumors (Wen 1995, Hazan-Halevy 2010). The transcriptional targets of activated STAT3 promote cell proliferation, survival (through inhibition of apoptosis), invasion, and angiogenesis (Schindler 2007). In normal cells, additional targets upregulated by STAT3, such as SOCS3, provide a negative feedback mechanism that inhibits the signal transduction cascades allowing the cell to return to steady state subsequent to the initial response to triggering factors (Sweeney 2002, Saxena 2007).

Our analysis of the effect of treatment of CRL2091 fibroblast and HT1080 fibrosarcoma cells with leptin (Fig 1) indicates that whereas both responded with quick upregulation of phosphorylation of STAT3 at the tyrosine residue at position 705, which is necessary for dimerization and nuclear localization of STAT3, the response of the fibroblast cells was initially

greater but attenuated to some extent over time. However, phosphorylation of STAT3 at Tyr 705 remained at the elevated level in the fibrosarcoma cells, possibly indicating impairment of regulatory mechanisms such as those involving SOCS3. The smaller amplitude of activation of the HT1080 fibrosarcoma cells may also be indicative of the extent of pre-existing constitutive activation of Tyr705 in these malignant cells. At the Ser 727 residue, increased phosphorylation was only displayed in the CRL2091 cells (Fig 2), possibly due to constitutive phosphorylation of the site in HT1080 fibrosarcoma cells.

ERKs are extracellular signal-related kinases, often known as classical MAP kinases, which are also activated via phosphorylation. ERKs have multiple isoforms and are typically involved in mitosis and meiosis, cell transformation, epithelial to mesenchymal transition, and invasion (Boulton 1991, Santamaria 2010). Activation of ERK may be triggered by the action of growth factors, cytokines, and viruses (Santamaria 2010). The ERK signaling pathway is constitutively upregulated in a variety of tumor tissues (Tanimura 2009). In our experiments, both HT1080 and CRL2091 cells displayed upregulation of pERK upon treatment with leptin (Fig 3) although the increase was 10 fold in CRL2091 compared to the 1.5 fold of HT1080. The results of other experiments conducted at the Kanjilal laboratory confirm that pERK was 4 fold more prevalent in HT1080 cells as compared to CRL2091 indicating preexisting activation (and hence a lower apparent response) in the HT1080 cells.

AKT, also known as protein kinase B, functions to inhibit apoptosis and promote cell survival, protein synthesis, and tissue growth during cancer development in a variety of cancer tissues including lung cells, T cells, breast cancer cells, and prostate cancer cell lines[3] (Staal 1977, Garofalo 2003, Yang 2004). Leptin has been demonstrated to increase AKT phosphorylation, leading to apoptosis resistance in rat renal tubular cells (Chen 2011). Both

[3] A kinase is any enzyme that catalyzes the transfer of an inorganic phosphate group from ATP to a specified molecule in order to activate or deactivate it.

fibroblast (CRL2091) and fibrosarcoma (HT1080) cells showed minimal alteration of AKT phosphorylation in the time interval of our experiments although the levels did possibly rise fractionally on longer-term leptin exposure (ongoing research).

Overall, our studies provide the initial steps towards elucidating the differences and similarities between responses of normal and malignant mesenchymal cells to the elevated leptin levels found in obese individuals. This research has significant implications in illuminating the relationship between the mechanisms linking obesity and fibrosarcoma. The knowledge gained from these studies will help in understanding the importance of leptin in the development of chronic conditions associated with obesity and diabetes. Ongoing work at the laboratory is focused on unraveling epigenetic alterations caused by exposure to elevated levels of leptin and their mechanistic role in modulating the signaling cascades and resultant phenotypic changes in normal and malignant cells.

APPENDIX A

Abbreviations

AKT- Protein Kinase B, Serine/Threonine Kinase

BSA- Bovine Serum Albumin

DMEM- Dulbecco's Modified Eagle Medium

ERK- Extracellular Signal-Regulated Kinase, or classical MAP Kinase

FBS- Fetal Bovine Serum

JAK- Janus Kinase

M-PER- Mammalian Protein Extraction Reagent

MAPK- Mitogen-Activated Protein Kinase

MMPs- Matrix Metalloproteinases, or Tissue Modeling Enzymes

PBS- Phosphate Buffered Saline

PVDF- Polyvinyldiene Difluride

SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

STAT- Signal Transducers and Activators of Transcription

TIMPs- Tissue Inhibitors of Metalloproteinases

VEGF- Vascular Endothelial Growth Factor

APPENDIX B

Materials

Normal foreskin fibroblasts CCD-1070Sk (CRL2091) and epithelial fibrosarcoma HT1080 cells were obtained from American Type Culture Collection (Manassa, VA).

Tissue culture medium (Dulbecco's Modified Eagle Medium, DMEM), phosphate buffered saline (PBS), trypsin, antibiotics and antimycotics (Streptomycin, penicillin, and amphotericin B) were obtained from Life Technologies (Grand Island, NY). Fetal Bovine Serum (FBS) was obtained from HyClone® (Thermo Scientific, Rockford, IL).

Primary antibodies for GAPDH, phospho-p44/42 MAPK (phosphorylated ERK1/2), p44/42 MAPK (ERK1/2), and phospho-AKT (AKT1/2/3 phosphorylated at Ser 473) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies for phospho-STAT3 (Ser 727), phospho-STAT3 (Tyr 705), and AKT 1/2/3 were obtained from Santa Cruz Biotechnology (Dallas, TX).

Mammalian Protein Extraction Reagent (M-PER) was obtained from Thermo Scientific. Complete Mini Protease Inhibitor Cocktail tablets were obtained from Roche Applied Science (Mannheim, Germany), and the Bradford protein assay kit was purchased from Bio-Rad (Hercules, CA). Bovine Serum Albumin (BSA) for preparation of a standard curve with the protein assay reagents was also procured from Bio-Rad.

Laemmli sample buffer, beta-mercaptoethanol, and Precision Plus Protein Prestained Standards were obtained from Bio-Rad. PVDF membranes were obtained from Millipore (Billerica, MA). Chemiluminescence western blot substrate kits such as SuperSignal West Pico, Dura, and Femto Chemiluminescent Substrate, Restore Plus Western Blot Stripping Buffer, and X-ray fim were obtained from Thermo Scientific. Other reagents required for Western Blotting

were made in house: (i) Tris-glycine SDS-polyacrylamide gel running buffer was prepared by dissolving 3.03g of Tris base, 14.4g of glycine, and 1g of SDS in 1L of MiliQ water; (ii) Tris-glycine blotting buffer was made by dissolving 3.03g of Tris base and 14.4g of glycine in 200ml of methanol and 800ml of MiliQ water.

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Education

- Penn State University, University Park, PA
- B.S. Premedicine
- Schreyer Honors College

Scholarships and Awards

- Schreyer Honors College Scholar, 2009-present
- Dean's List, Fall 2010, Spring 2011, Spring, Summer, and Fall 2012

Areas of Research Interest

- Effects of diet and lifestyle on cancer development, progression, and prevention
- Development, treatment, and prevention of pediatric cancers

Academic-Related Experience

- Volunteer and On-Site-Coordinator for Student Red Cross Club, 2009-present
- Volunteer at Mount Nittany Medical Center, 2011-2012
- Assistant Teacher at Penn Mont Academy, 2008-2010
- Volunteer and tutor at Hollidaysburg YMCA, 2009

Software Programs

- Microsoft Word, Microsoft PowerPoint, Microsoft Excel, ImageJ Densiometry Analysis

Other Activities/Interests

- Member of Penn State Thespians, 2009-present
- Pianist for charity productions, MasquerAIDS and Harmonies for Hope, 2009-present
- Volunteer for Penn State IFC/Panhellenic Dance MaraTHON, 2009-present
- Chairperson for Penn State Thespians benefitting THON, 2011-2012
- Proficient artist in various mediums including graphite, charcoal, oils, acrylics, ink, and clay