

Selective Activation of Insulin Receptor Substrate-1 and -2 in Pleural Mesothelioma Cells: Association with Distinct Malignant Phenotypes

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ABSTRACT

Molecular mechanisms active in transforming human pleural cells remain incompletely understood. Our previous microarray analysis of malignant pleural mesothelioma revealed alterations in components of the insulin-like growth factor (IGF) system, implicating this signaling axis in tumorigenesis. Therefore, in this current study, we characterized the molecular phenotype and investigated the key signaling pathways of the IGF system in malignant pleural mesothelioma specimens. For the major IGF components, we assessed mRNA abundance and total protein levels. We measured IGF-I ligand-dependent activation of signaling pathways downstream of the type I IGF receptor in a subset of malignant pleural mesothelioma cell lines and determined the corresponding biological consequences. At the transcriptional level, we observed consistent changes in IGF components that may contribute to a malignant phenotype. IGF-I stimulation of cells resulted in enhanced activation of type I IGF receptor and IRS adaptor proteins. Differential activation of IRS-1 signaling was associated with cell growth, whereas IRS-2 signaling was associated with cell motility. Thus, these data suggest that multiple mechanisms likely contribute to malignant pleural mesothelioma tumorigenesis. Therefore, IGF system components represent novel malignant pleural mesothelioma therapeutic targets for investigation.

INTRODUCTION

Malignant pleural mesothelioma is an aggressive neoplasm of the serosal lining of the pleural cavity arising from mesothelial cells (*i.e.*, from undifferentiated cells representing the adult remnants of the surface coelomic mesoderm). Mesothelial cells are biphasic, giving rise to three major forms of malignant pleural mesothelioma: epithelial, sarcomatoid or fibrous, and biphasic. Currently, malignant pleural mesothelioma accounts for ~2,500 to 3,000 deaths per year in the United States (1). Up to 80% of cases of malignant pleural mesothelioma occur in patients 10 to 20 years after exposure to asbestos (2). Other factors, such as ionizing radiation or a tumor DNA virus known as SV40, may act synergistically in malignant pleural mesothelioma pathogenesis (3, 4). Also, several well-defined acquired genetic targets have been identified in malignant pleural mesothelioma, including the 9p21 (p16^{INK4a} and p14^{ARF}) and the 22q11-q13.1 loci (NF2; ref. 2).

However, the molecular mechanisms regulating the transformation of mesothelial cells remain poorly defined, as evidenced by the relative paucity of effective, specific chemotherapeutic regimens (5). Moreover, these well-characterized causes incompletely account for the known incidence of malignant pleural mesothelioma. About 10%

to 20% of all malignant pleural mesothelioma cases have been documented in patients without previous exposure to asbestos (2); only 60% of malignant pleural mesothelioma tumors are known to contain SV40 viral DNA (4). Accordingly, multiply active tumorigenic pathways are thought to be possible.

To identify novel candidate genetic targets, we recently performed a cDNA microarray profiling study of both malignant pleural mesothelioma cell lines ($n = 10$) and tissues ($n = 4$) versus nonmalignant mesothelium (6). Notably, we found consistent overexpression of transcripts for insulin-like growth factor (IGF)-I and underexpression of transcripts for IGF binding protein (IGFBP)-5, both relative to nonmalignant mesothelial cells. That finding suggests an alternate oncogenic pathway. In studies of multiple solid tumors, especially breast cancer (7), IGF signaling via a complex network of molecules clearly contributes to a malignant phenotype by enhancing cellular proliferation and protection from apoptosis.

The ligands (IGF-I and IGF-II), well-characterized polypeptide mitogens, have been implicated as both autocrine and paracrine growth factors (8). That effect is mediated by the type I IGF receptor (IGFR1), a transmembrane, heterotetrameric protein with tyrosine kinase activity. When activated, the receptor phosphorylates multiple classes of signal transduction adaptors: IRS, src-homology 2 (Shc), Crk, or growth factor receptor-binding protein 10, and others (9). In turn, those signal transduction adaptors activate numerous downstream signaling pathways, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3'-kinase (9). In solid tumors such as breast cancer, the predominant adaptor proteins are the IRS-1 and IRS-2 proteins (10). The IGF binding proteins (IGFBP-1 to -6) bind the IGF ligands in biological fluids and serve to modulate IGF-I actions (11).

In comparison, much less is known about the precise role of the IGF system in malignant pleural mesothelioma tumorigenesis; a large proportion of the limited functional data are from animal models. For example, one study showed that mouse embryonic fibroblasts lacking IGFR1 (because of a targeted gene disruption) were resistant to transformation by SV40 large tumor antigen and that a transformed phenotype is restored to such cells after stable transfection with a plasmid expressing human IGFR1 (12). That study found that IGFR1 signaling is necessary for growth and transformation of nonmalignant cells, albeit in nonhuman cells. Similar experiments using human malignant pleural mesothelioma cells are very few in number. In one study, gel-based, reverse transcription-PCR was used to qualitatively estimate transcript levels of IGF-I, IGFR1, and the major IGFBP in a panel of malignant pleural mesothelioma cell lines derived from human patients (13). That study showed increased proliferation of nonmalignant, human mesothelial cells in response to exogenous IGF-I. Thus, the cumulative data from both animal and human experiments suggest that the IGFR1 mediates the mitogenic signaling of IGF-I in malignant pleural mesothelioma, likely contributing to tumorigenesis. However, the critical IGFR1 signaling pathways remain undefined in human malignant pleural mesothelioma and so do the phenotypic effects resulting from activation of those pathways.

A detailed identification of the specific molecules involved in IGF

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signaling in malignant pleural mesothelioma could have significant therapeutic implications. In our current study, our objectives were to determine, in human malignant pleural mesothelioma specimens, whether or not the IGF system was dysregulated at the transcript level, to determine which signaling molecules become aberrantly activated by IGF-I, and to correlate pathway activation states with biological responses. In a representative sampling of malignant pleural mesothelioma cell lines and tissues, we quantified transcripts of the major IGF components, using quantitative real-time PCR. For a subset of malignant pleural mesothelioma cell lines, we extended those results by examining the concurrent protein expression levels by IGF species-specific immunoblotting. For certain malignant pleural mesothelioma cell lines, *in vitro* IGF-I-induced growth and migration confirmed the observed mRNA and protein profiles.

MATERIALS AND METHODS

Specimens and Reagents. As detailed in our previous report (6), we studied a total of 10 cell lines (14) and 4 primary tumor specimens (Table 1). The SV40-transformed mesothelial cell line CRL-9444 served as the baseline reference for our quantitative real-time PCR experiments. The breast cancer cell line MCF-7 served as a positive control (15) for our IGF transcript and protein expression studies. Both of those cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in the appropriate medium according to instructions.

All of the chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. IRS-1, IRS-2, MAPK, and phospho-specific IGFR1 primary antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Serine-threonine kinase (Akt), phospho-specific Akt, and IGFR1 (β -subunit) primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The status of Akt protein expression and activation served as a surrogate marker for phosphatidylinositol 3'-kinase signaling, because Akt lies immediately downstream of phosphatidylinositol 3'-kinase (9). Horseradish peroxidase-conjugated PY-20 antiphosphotyrosine primary antibody was purchased from Transduction Laboratories (Lexington, KY). Phosphospecific MAPK primary antibody was purchased from New England Biolabs (Beverly, MA). Horseradish peroxidase-conjugated antirabbit or antimouse secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ). IGF-I was purchased from GroPep Limited (Adelaide, Australia).

RNA Isolation. Cells growing asynchronously were lysed in Trizol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) when they reached ~70% confluence and then processed as described previously (6). Briefly, for total RNA extraction, the Trizol method was coupled with a silica gel-based membrane spin column (Qiagen, Valencia, CA) per the manufacturer's instructions. On-column DNase digestion was performed with all of the samples to eliminate potential genomic DNA. RNA yield and purity were determined by spectrophotometry. Integrity was verified on 1.5% agarose-formaldehyde

Table 2 Probe and primer pair sequences specific for IGF component transcripts

IGF components	Probe and primer pair sequences
IGF-I	Probe: 6FAM-CGTTTCCGATTGCTGGCCGTAGTTCT-TAMRA Forward: 5'-GCTGTCAGTGCACCGACTTC-3' Reverse: 5'-GGATATAATTTTGTGAGAATGGAAAC-3'
IGF-II	Probe: 6FAM-TGGCCTTCGCCTCGTGC-TAMRA Forward: 5'-GATGCTGGTGTCTTCCACCTT-3' Reverse: 5'-GGCGGTAAGCAGCAATGC-3'
IGFR1	Probe: 6FAM-CCTCCACCACCGTCGAAAGAATC-TAMRA Forward: 5'-TGAAAGTGACGTCCTGCATTTC-3' Reverse: 5'-CGGTGCCAGGTTATGATGATG-3'
IGFR2	Probe: 6FAM-TGGTGAAAATAAGACCGCATCTCCG-TAMRA Forward: 5'-TGTCACGTACTCCAAGGTTATCC-3' Reverse: 5'-CCACCGTCTTTGTACAGGTCAA-3'
IRS-1	Probe: 6FAM-AGGTGGGCTTCTGCGGTG-TAMRA Forward: 5'-CCACTCGGAAAACCTTCTTTCAT-3' Reverse: 5'-GAGTCATCCACCTGCATCCA-3'
IRS-2	Probe: 6FAM-AGGAGGCCACCATCGTAAAGAGTGAAG-TAMRA Forward: 5'-CTACGCCAGCATGACTTCTTGT-3' Reverse: 5'-GACATGTGACATCTGGTGATAAAG-3'
IGFBP-1	Probe: 6FAM-TTCCAAATTTTACCTGCCAACTGCAACA-TAMRA Forward: 5'-CACAGGAGACATCAGGAGAAGAAA-3' Reverse: 5'-ACTGTCTGCTGTGATAAAATCCATTC-3'
IGFBP-2	Probe: 6FAM-CCCCAAGTTCACCAAGCATGGC-TAMRA Forward: 5'-GCCCTCTGGAGCACCTTACT-3' Reverse: 5'-TCTTGCACCTGTTGAGGTGTGACA-3'
IGFBP-3	Probe: 6FAM-CCCAAGTTCACCAAGCATGGC-TAMRA Forward: 5'-ACGCACCGGTGTCTGA-3' Reverse: 5'-TGCCTTTCTTGATGATGATTATC-3'
IGFBP-4	Probe: 6FAM-CGCCATGACCGCAGGTGC-TAMRA Forward: 5'-ACAACAGCTTCAGCCCTGTA-3' Reverse: 5'-CGAATTTTGGCGAAGTGCTT-3'
IGFBP-5	Probe: 6FAM-CTGGCTTCCAAGAGAAGGAGGCT-TAMRA Forward: 5'-CCCATAGCGTCAGAGAGGATATG-3' Reverse: 5'-TCCTCTCCACTTTGCAAA-3'
IGFBP-6	Probe: 6FAM-CCCAAGCATGGCCTGCGC-TAMRA Forward: 5'-GCAGAGGAGAATCCTAAGGAGAGTAA-3' Reverse: 5'-TGGTCTTGCAGTTTACATC-3'

gels stained with ethidium bromide. Tissue specimens were processed in a similar fashion, each starting from 100 mg of frozen tumor.

Real-Time PCR. To characterize the transcript profile of IGF components in malignant pleural mesothelioma and to confirm the findings from our previous microarray study, we determined the quantitative expression of the 12 main IGF genes in 14 malignant pleural mesothelioma specimens by quantitative real-time PCR, according to protocols described previously (16). We designed TaqMan probe and primer sets from the open reading frame of IGF genes (Table 2) using Primer Express software v2.0 (Applied Biosystems, Foster City, CA). The TaqMan probe and primer sequences were reported previously for the endogenous control gene β -glucuronidase (*GUSB*; ref. 17), which we used for normalization of sample loading. PCR products of the expected size for each gene were eluted from ethidium bromide-stained 2% agarose gels; their identities were confirmed by sequence analysis.

Relative quantitation of IGF component transcripts was calculated by the comparative threshold cycle method (18, 19). To facilitate comparison between the different techniques, we chose CRL-9444 for the quantitative real-time PCR calibrator; this was the same reference sample used in all of our microarray hybridizations. For all of the samples, we calculated the relative, normalized quantitative expression level for each IGF gene ($2^{-\Delta\Delta\text{threshold cycle}}$).

Cell Stimulation and Lysate Preparation. For six selected malignant pleural mesothelioma cell lines (Table 1), we conducted stimulation experiments in triplicate, as described previously (15). Briefly, cells were cultured in RPMI 1640 (Invitrogen Life Technologies, Inc.) supplemented with 10% fetal calf serum and antibiotics until 70% confluent, then washed twice with warmed PBS (Biofluids, Rockville, MD). Medium was then changed to serum-free medium, consisting of Improved Minimal Essential Medium with Richter's modification (Invitrogen Life Technologies, Inc.) and other supplements (15) for 24 hours and then replaced with serum-free medium plus 5 nmol/L IGF-I for 10 minutes. That IGF-I stimulation time was established after exposing our panel of malignant pleural mesothelioma cells to IGF-I for a series of different times (3, 10, and 20 minutes) and assessing which was optimal (data not shown). Cells were washed twice in ice-cold PBS and lysed with 500 μ L per 10-cm plate with TNESV buffer (50 mmol/L Tris (pH 7.4), 1% NP40, 2 mmol/L EDTA, 100 mmol/L NaCl, 10 mmol/L sodium orthovana-

Table 1 Pathologic characteristics of mesothelioma specimens used in gene expression profiling

Specimens	Histologic type
CRL-9444 (MeT-5A)	Mesothelium, epithelial
CRL-2081 (MSTO-211H)	Biphasic
NCI-H28	Epithelial
NCI-H513*	Epithelial
NCI-H2373*	Sarcomatoid
NCI-H2452	Biphasic
NCI-H2461*	Epithelial
NCI-H2595*	Epithelial
NCI-H2596*	Sarcomatoid
NCI-H2618*	Epithelial
NCI-H2691	Epithelial
MPM 1	Epithelial
MPM 2	Epithelial
MPM 3	Epithelial
MPM 4	Biphasic

Abbreviation: NCI, National Cancer Institute.

* The IGF system was additionally characterized in these cell lines for our study.

date, 1 mmol/L phenylmethylsulfonylfluoride, 20 mg/mL leupeptin, and 20 mg/mL aprotinin). Protein concentration was determined by the Bradford dye-binding protein assay (Bio-Rad Laboratories, Hercules, CA). Total protein (50 μ g) lysates were resolved by 7.5% SDS-PAGE.

IRS Immunoprecipitation. All of the steps were performed on a platform rocker at 4°C. Equal amounts of protein from H2373 and H2461 cell lines (500 μ g) were first precleared with 25 μ L of protein A-agarose (Pierce, Rockford, IL) for 30 minutes, then incubated overnight with 1 μ L IRS-1 or IRS-2 antiserum per 100 μ g of cellular protein. Protein A-agarose of 25 μ L was then added for 4 hours followed by three washes with TNEVS buffer. Beads were resuspended in an equal volume of TNEVS and 2 \times Laemmli loading buffer with 30 mg/mL dithiothreitol and boiled briefly. The resultant protein lysate was resolved by 7.5% SDS-PAGE.

Immunoblotting. After SDS-PAGE, proteins were transferred overnight at 4°C onto nitrocellulose membranes (Bio-Rad Laboratories). All of the blotting steps were carried out at room temperature with gentle rocking. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline-Tween 20 [0.15 mol/L NaCl, 0.01 mol/L Tris-HCl (pH 7.4) and 0.05% Tween 20] followed by Tris-buffered saline-Tween 20 washing (six times) over a period of 30 minutes. Antiphosphotyrosine blotting with the PY-20 antibody was performed over a period of 1 hour at a dilution of 1:5,000 without requiring a secondary antibody incubation. IGFR1, phospho-specific IGFR1, Akt, and phospho-specific Akt blotting was performed with antibodies diluted 1:1,000, according to manufacturer instructions. Similarly, IRS-1, IRS-2, MAPK, and phospho-specific MAPK blotting was performed with antibodies diluted 1:2,000. After incubation for 1 hour in primary antibody, blots were washed and then incubated with a 1:4,000 dilution of an appropriate horseradish peroxidase-linked secondary antibody in 2.5% blocking buffer for 1 more hour. A final wash preceded visualization of protein bands by enhanced chemiluminescence detection, per the manufacturer's instructions (Pierce).

Cell Proliferation Assay. Cells in serum-containing medium were seeded as triplicate sets into 24-well plates with 20,000 cells per well. Cells were switched to serum-free medium for 24 hours, then treated with or without 5 nmol/L IGF-I as described previously (15). Cell growth was assayed 5 days after treatment by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (20). Briefly, 60 μ L of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution in PBS was added to each well. After incubation for 4 hours at 37°C, wells were aspirated, and formazan crystals were lysed with 500 μ L of solubilization solution (95% DMSO + 5% Improved Minimal Essential Medium). Absorbance was measured with a plate reader at 570 nm by using a 670-nm differential filter.

Cell Migration Assay. Cell motility was assessed with a modified Boyden chamber assay (21). Cells in serum-containing medium were detached by trypsin (brief incubation), washed twice with PBS, and then resuspended in serum-free medium. In the upper chambers of a 10-well Boyden apparatus (NeuroProbe, Gaithersburg, MD), 150,000 cells were placed. Upper and lower chambers were separated by a polycarbonate polyvinylpyrrolidone-free filter with 12- μ m pores. Serum-free medium with or without 5 nmol/L IGF-I was placed in the bottom chambers. After incubation for 4 to 6 hours, cells remaining on the topside of the filter were removed with cotton swabs. Cells that had migrated to the underside of the filter were fixed and stained in HEMA3 (Fisher Scientific, Hampton, NH). The filter was mounted onto a glass slide, and cells were counted in five different areas by using a light microscope.

Statistics. Analysis was performed on S-Plus v6.1 software (Insightful, Seattle, WA). We compared differences of gene expression results between microarray and quantitative real-time PCR techniques according to methods described previously (22). Briefly, we determined the distribution of log-transformed gene expression ratios (tumor specimen/reference sample) for IGF components. We assessed whether the means of the gene expression ratios were statistically similar by using a two-tailed Student's *t* test with level of significance set at $P < 0.05$. Where appropriate, data are presented herein as the mean \pm 1 SD.

RESULTS

Aberrant Expression of IGF Genes. Our initial microarray analysis, as in other previous studies, implicated the IGF system in malignant pleural mesothelioma tumorigenesis. We re-examined our microarray data specifically for genes of the IGF family and identified

4 additional ones that were represented on the cDNA microarray: *IGFR1*, *IRS-2*, *IGFBP-3*, and *IGFBP-6*. The overall pattern of gene expression based on the 6 IGF component genes additionally implied the importance of this system in maintaining the malignant phenotype of malignant pleural mesothelioma. Using quantitative real-time PCR to test the identical malignant pleural mesothelioma cell lines and tissues for transcript abundance of all 6 of the IGF genes, we additionally validated our previous microarray data. In our current study, the quantitative real-time PCR-derived mean expression ratio changed in the same direction of differential expression as predicted by our microarray analysis (data not shown). Student's *t* test confirmed that the mean expression ratios for 5 of 6 genes (except *IGFBP5*), as calculated by microarray versus quantitative real-time PCR, were not statistically different ($P > 0.05$). Thus, results for each gene were broadly consistent between the two different techniques.

On the basis of these confirmatory findings, we quantitated transcript abundance of additional components of the IGF system (12 in all) relative to the mesothelial cell line CRL-9444 to comprehensively characterize the IGF gene profile in malignant pleural mesothelioma. In particular, we found that mRNA transcripts were significantly overexpressed (overall mean value) in our malignant pleural mesothelioma specimens for IGF-I by 43.3-fold, for IGFR1 by 2.6-fold, for IRS-1 by 4.4-fold, and for IRS-2 by 24.0-fold (Fig. 1). Because SV40 may also induce expression of IGF-I and IGFR1 (12), our results may

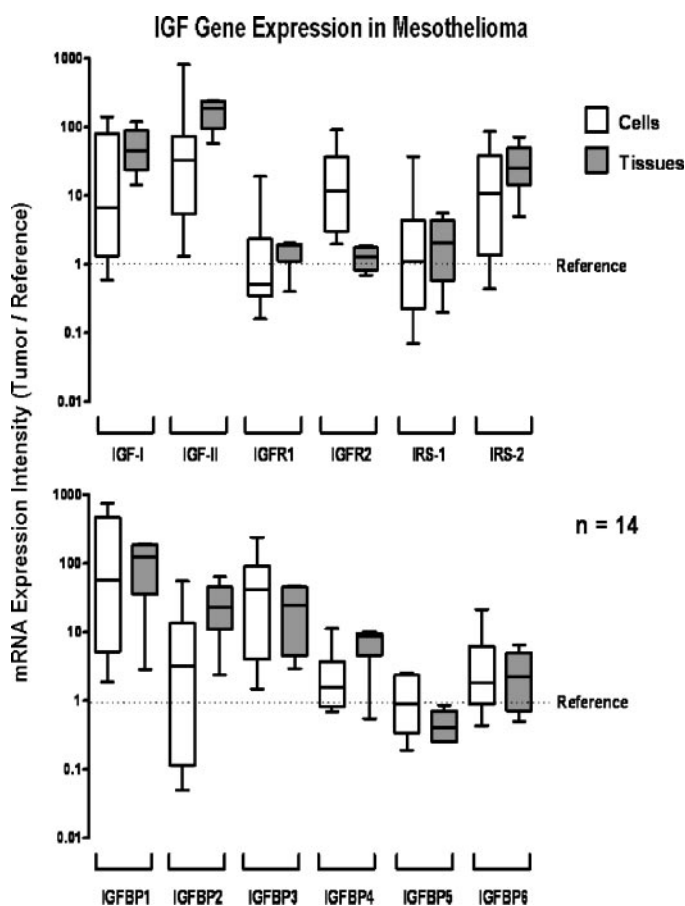


Fig. 1. Depicts mRNA transcript profile of the IGF system in mesothelioma. IGF-species-specific IGF-I, -II, IGFR1, -2, IRS-1, -2, and IGFBP-1 to -6) TaqMan probe and primer sets were designed. Then mRNA levels of each IGF gene from 14 mesothelioma specimens (10 cell lines and 4 tissues) were quantitated by using the comparative threshold method ($2^{-\Delta\Delta C_t}$) and expressed as fold change (on a logarithmic scale) relative to a nonmalignant mesothelial cell line, CRL-9444 (Reference). Top panel, differential expression of IGF-I, IGF-II, IGFR1, IGFR2, IRS-1, and IRS-2. Bottom panel, differential expression of the IGFBP proteins. Results are grouped according to cell line (white) and tumor tissues (gray).

be an underestimate of IGF transcript alterations in malignant pleural mesothelioma. The cumulative transcript level changes in malignant pleural mesothelioma specimens that we observed for the IGFBP may potentiate IGF-I tumorigenic action, as suggested by the literature (11). Thus, aside from additional post-translational mechanisms, alterations at the transcript level in our current study suggest corresponding changes of IGF component protein expression.

Response of IGF Component Proteins to IGF-I Stimulation. To determine the significance of altered IGF gene expression, we initially assessed the pattern of protein expression and then the response of IGFR1 to exogenous IGF-I stimulation in a subset of malignant pleural mesothelioma cell lines. Immunoblotting showed that IGFR1 protein levels (transmembrane β subunit) were variable across 6 malignant pleural mesothelioma cell lines; 3 cell lines expressed high levels of IGFR1 similar to MCF-7, a cell line known to overexpress IGF components (Fig. 2A). Treatment of cells with IGF-I for 10 minutes clearly activated the IGFR1 (relative increased autophosphorylation from the non-IGF-I-treated specimen) in 4 cell lines (H2461, H2618, H2596, and H513). Thus, we showed that the key receptor responsible for IGF signaling was functionally expressed to a variable extent in malignant pleural mesothelioma cells.

Next, to confirm that IGF-I-dependent activation of IGFR1 ultimately leads to transduction of critical signaling pathways in malignant pleural mesothelioma cells, we needed to determine which class of signal trans-

duction adaptor proteins (immediately downstream of IGFR1) is activated. Shc was reported previously to interact directly with IGFR1 and stimulate tumorigenesis in other cancer types (23). Shc protein expression was undetectable, however, in our panel of malignant pleural mesothelioma cell lines by Shc-specific immunoblotting (data not shown). Therefore, our findings suggest that the Shc signaling pathway is not important in malignant pleural mesothelioma.

We then proceeded to screen our malignant pleural mesothelioma cells for protein expression of IRS species. Total levels of IRS-1 and IRS-2 varied across our 6 malignant pleural mesothelioma cell lines (Fig. 2B). Notably, H2373 cells showed high protein expression of IRS-1 and low protein expression of IRS-2, whereas H2461 cells showed the inverse of this IRS protein profile. We used total antiphosphotyrosine immunoblotting to assess the activation status of IRS species, because IRS-1 and IRS-2 are typically known to have a molecular weight of about M_r 190,000 in that type of assay. In our study, IGF-I-stimulated cells showed activation of IRS species similar to a positive control, clearly seen in cell lines H2595, H2373, and H2461; for specimen H2618, the effect is less noticeable (Fig. 2C). To confirm specific activation of IRS subtypes by IGF-I, we immunoprecipitated IRS-1 and IRS-2 from cell lysates and then performed antiphosphotyrosine immunoblotting. In H2373 cells, IRS-1 was the major IRS subtype tyrosine phosphorylated by IGF-I; in contrast, IRS-2 was

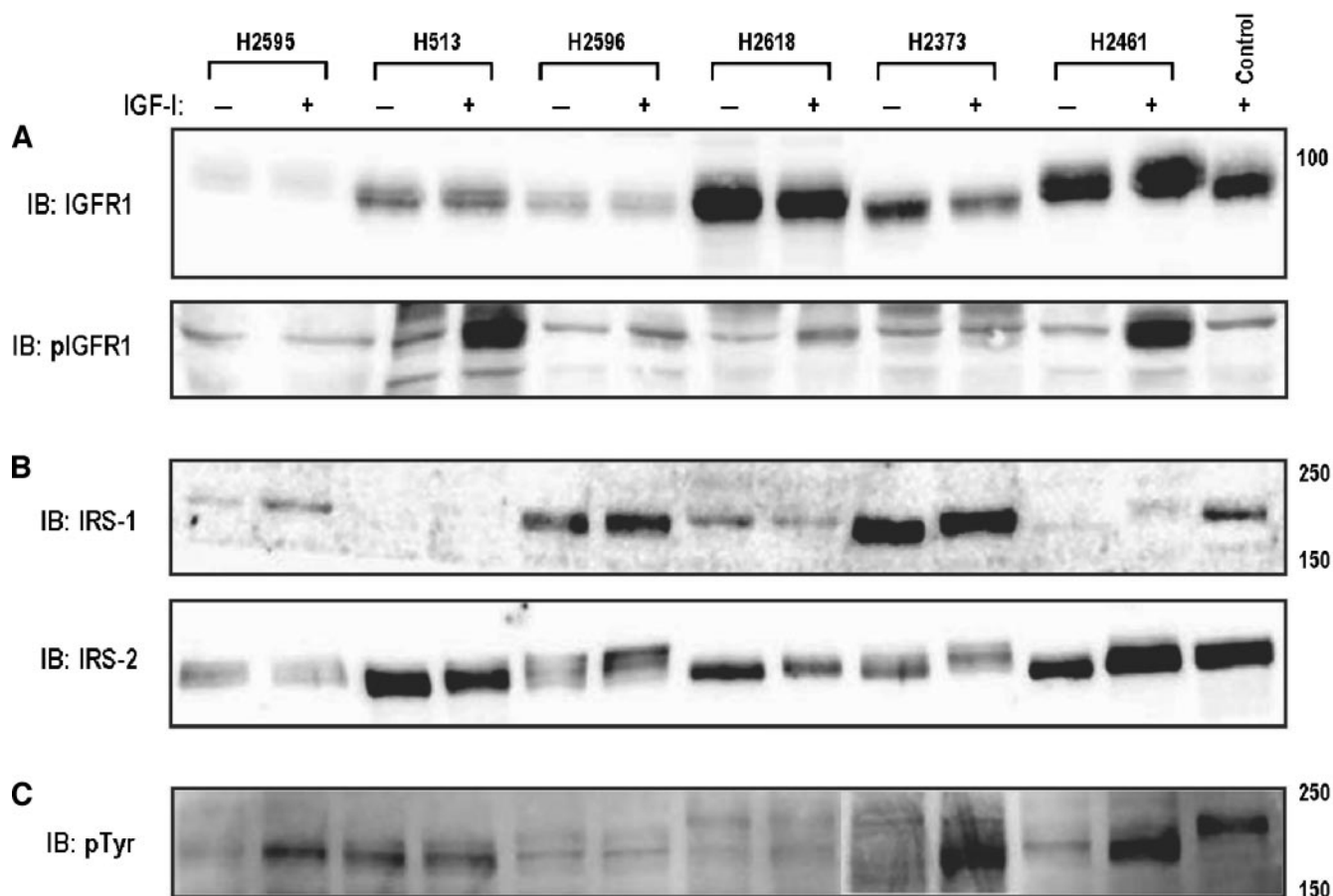
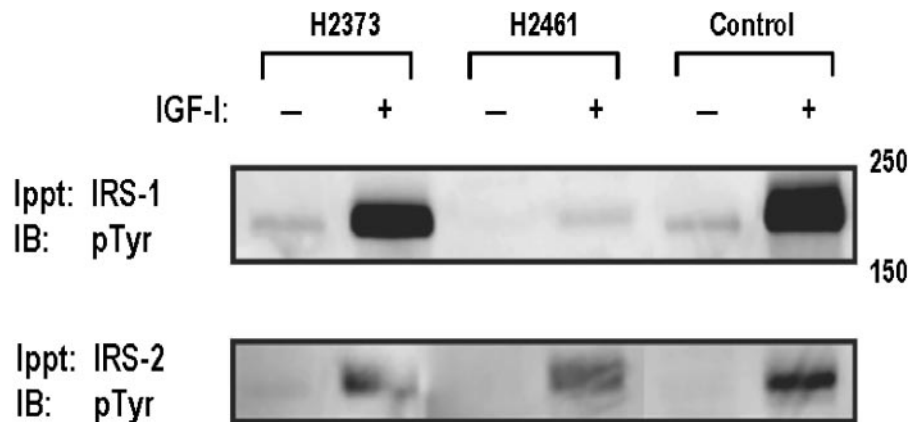


Fig. 2. Depicts IGFR1 and IRS species protein expression and activation in response to IGF-I stimulation. In every lane, 50 μ g of total protein from 6 MPM cell lines were resolved by SDS-PAGE and then analyzed by IB. A, total IGFR1 protein level in each sample determined by an anti-IGFR1 β subunit-specific monoclonal antibody (top). Similarly, a phospho-specific IGFR1 (pIGFR1) antibody was used to assess increased phosphorylation, which is a marker for relative activation (bottom). B, total levels of IRS-1 (top) and IRS-2 (bottom) proteins in each sample determined by IRS species-specific monoclonal antibodies. C, total antiphosphotyrosine (pTyr) antibody was used to detect activation (increased phosphorylation) in the region of IRS species migration on an immunoblot. Cells were either treated with SFM and 5 nmol/L IGF-I (+) or exposed only to SFM (-). Experiments were performed in triplicate. Representative blots are shown with molecular weight markers to the right. Control = MCF-7 breast cancer cell line. (MPM, malignant pleural mesothelioma; IB, immunoblotting; SFM, serum-free medium.)

Fig. 3. Depicts the IRS subtype activated by IGF1 signaling. IRS-1 (*top*) and IRS-2 (*bottom*) proteins were Ippt from whole-cell lysates. Activation in response to IGF-I stimulation was determined by total antiphosphotyrosine immunoblotting (500 μ g of each sample). Representative blots are shown with molecular weight markers to the right. Control = MCF-7 breast cancer cell line (*Ippt*, immunoprecipitated).



activated to a similar degree in H2373 and H2461 cells (Fig. 3). Taken together, those experiments indicate that H2373 cells mediate IGF-I action via IRS-1 and H2461 cells via IRS-2.

IGF-I-Mediated Signaling Pathways. MAPK and phosphatidylinositol 3'-kinase (Akt) are well-characterized, critical pathways downstream of IRS proteins that contribute to a malignant phenotype in breast cancer (10). To ascertain whether those pathways are important in malignant pleural mesothelioma, we assessed the activation

state of MAPK and Akt effector proteins by phosphospecific immunoblotting. Each malignant pleural mesothelioma cell line in our panel showed similar increased activity of both MAPK and Akt in response to IGF-I treatment (Fig. 4). Total MAPK blotting on the same membrane showed that levels of MAPK were equal in all of our malignant pleural mesothelioma specimen pairs (IGF-I treatment *versus* serum-free medium), indicating an IGF-I-specific effect. We observed identical results for total Akt blotting.

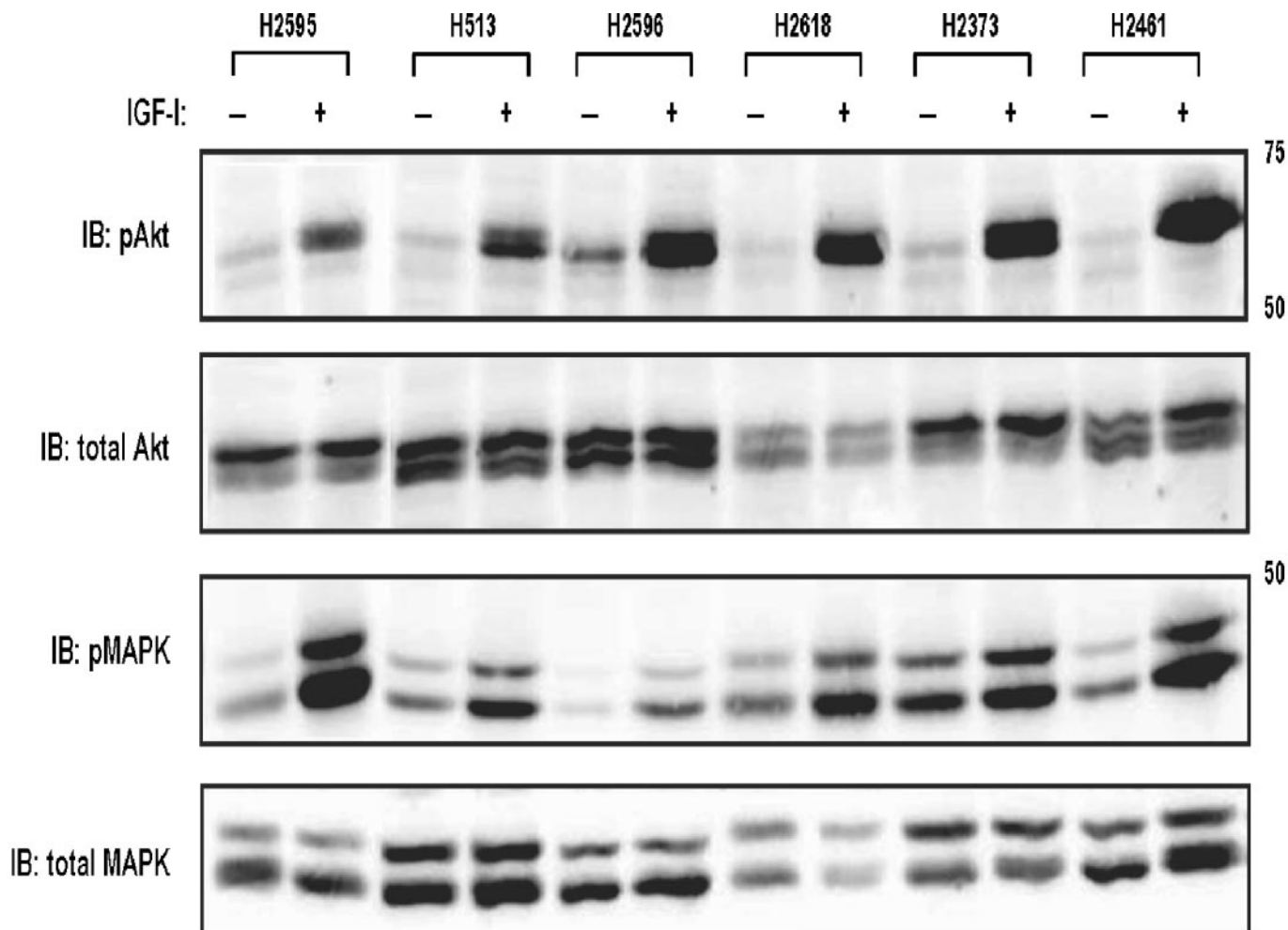


Fig. 4. Depicts activation of downstream signaling pathways in mesothelioma cells. In every lane, 50 μ g of total protein from 6 MPM cell lines were resolved by SDS-PAGE and then analyzed. Cells were incubated in the absence (-) or presence (+) of 5 nmol/L IGF-I. Phosphospecific Akt (*pAkt*) IB was performed. Total Akt immunoblotting was performed on a duplicate membrane to show equal sample loading between each cell pair. Analysis for MAPK activation was performed in an analogous manner. Experiments were performed in triplicate; representative blots are shown. (MPM, malignant pleural mesothelioma; IB, immunoblotting.)

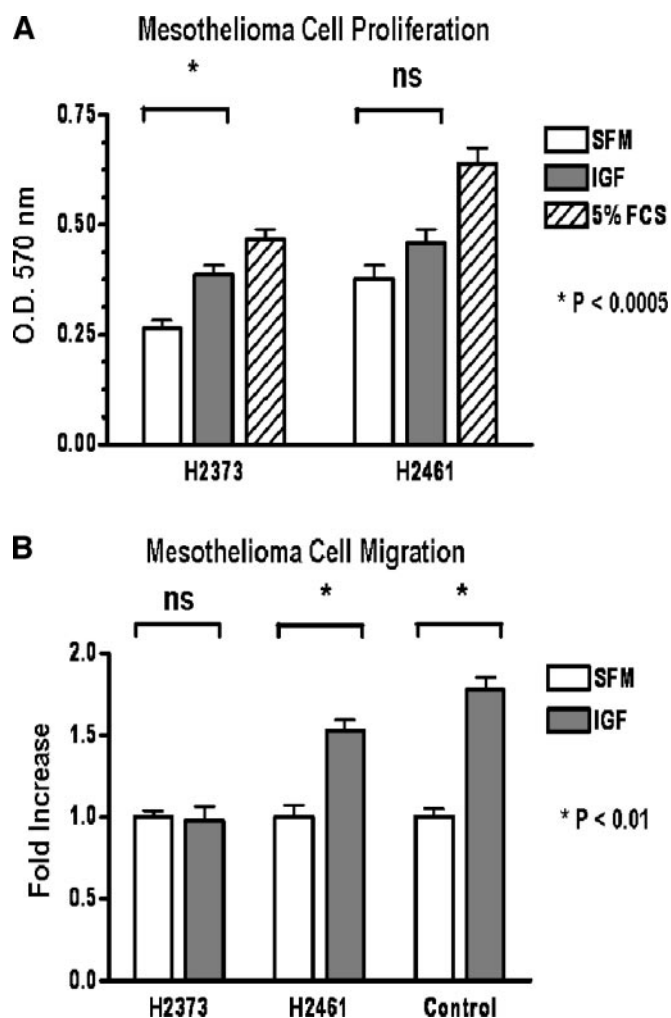


Fig. 5. Depicts cell growth and motility in response to IGF-I stimulation. A. Cells were placed into 24-well plates and allowed to grow in a monolayer in SFM only; in SFM supplemented with FCS; in SFM and 5 nmol/L IGF. After 5 days, cell numbers were estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. O.D. = absorbance as measured by a plate reader. B. Cells were placed in the top chamber of a Boyden chamber apparatus. SFM with or without 5 nmol/L IGF was placed into the bottom chamber; incubation lasted for 4 to 6 hours. Cells that had migrated across the porous membrane were counted. Results are expressed as fold increase over control (SFM). In both panels, bars, \pm SD of measurements from triplicate wells. (SFM, serum-free medium; FCS, fetal calf serum; IGF, IGF-I; ns, nonsignificant.)

Effects of IGF-I Activation of IRS Species. Our next series of experiments were aimed at determining the specific phenotypic effects on malignant pleural mesothelioma cells from IGF-I stimulation and which IGF molecules mediate those effects. We selected the 2 cell lines with opposite IRS protein profiles and monitored cell growth over a period of 5 days in the presence or absence of IGF-I (Fig. 5A). H2373 cells (high IRS-1 content) showed a more significant IGF-I growth response, as compared with H2461 cells (high IRS-2 content). Thus, our findings suggest that, in malignant pleural mesothelioma cells, IGF1R activation of IRS-1 is associated with cellular proliferation.

We used migration of cells across a porous membrane in a modified Boyden chamber as a functional assay for cell motility. In response to IGF-I, H2461 cells migrated through the membrane to a similar extent as a breast cancer cell line (21) with known motile behavior (Fig. 5B). IGF-I significantly enhanced motility in H2461 cells as compared with their unstimulated counterparts. H2373 cells did not show any enhancement of cell migration in response to IGF-I. Thus, our results suggest that IGF-I treatment can also induce cell migration, a biological effect associated with IRS-2 phosphorylation.

DISCUSSION

The IGF ligands, synthesized by a wide range of tissues in the body, are important mediators of normal cell growth, development, and survival (8, 9). Their pleiotropic biological actions are modulated by an intricate network of binding proteins, receptors, and signal transduction adaptors. Abundant evidence clearly indicates that when certain IGF components become dysregulated and aberrantly expressed, the IGF system may instead promote uncontrolled growth, evasion of apoptosis, motility, and metastasis (8, 24). The accumulating data indicate a significant role for the IGF system in the pathogenesis of an increasing number of solid tumors in breast, prostate, colon, or ovarian cancer, and, equally likely, in the maintenance of the malignant phenotype (8, 24). Our current study, based on tissues and cells, provides direct evidence to relate malignant pleural mesothelioma to the class of solid tumors affected by the IGF system.

Previously, our knowledge of the specific molecules and pathways involved in IGF signaling of human malignant pleural mesothelioma has been relatively incomplete as compared with other malignancies, such as breast cancer. Anecdotal observations from case reports investigating the phenomenon of extrapancreatic tumor hypoglycemia initially suggested that the IGF system was dysregulated in malignant pleural mesothelioma (25–29); the tumors elaborated high amounts of IGF-II into the serum, inducing hypoglycemia. In an effort to better understand the molecular mechanism for these findings, Hodzic *et al.* (30) analyzed a single such malignant pleural mesothelioma tumor for expression of IGF-II mRNA and protein levels, as well as the IGF1R and the major IGF1BP mRNA levels. Lee *et al.* (13) characterized the mRNA expression of IGF-I, IGF1R, and IGF1BP3 in 5 normal human mesothelial cell samples and in 11 malignant pleural mesothelioma cell lines, thus suggesting that the IGF system was potentially important in the development of malignant pleural mesothelioma. Lee *et al.* (13) also showed that normal mesothelial cells proliferated in response to exogenous IGF-I stimulation, thereby suggesting the presence of a functional IGF1R. Surprisingly, enhanced cell proliferation was not observed in 2 malignant pleural mesothelioma cell lines stimulated with IGF-I, and the authors did not offer additional explanation for this finding.

Our current experiments expanded on the previous studies investigating the IGF system in malignant pleural mesothelioma that were based on animal models and human specimens. We determined the quantitative changes of IGF transcripts in malignant pleural mesothelioma cells and tissues, as compared with a nonmalignant mesothelial cell line: we found overexpression of the IGF ligands, the IGF1R, and both IRS species. Overall, then, the increased expression of the IGF system, which is consistent with an autocrine growth loop, was permissive for tumorigenesis. We confirmed that observation by determining that critical proteins in the IGF signaling pathway were expressed in certain malignant pleural mesothelioma cells and became activated in response to exogenous IGF-I stimulation (based on the cumulative findings of immunoblotting experiments directed toward a specific molecule in the IGF signaling cascade). Significantly, we found evidence that IGF-I-induced cell proliferation was mediated by IRS-1, whereas IGF-I-induced cell migration was mediated by IRS-2. This observation appears to be consistent across different tumor cells such as breast (31) and melanoma.³

Our results on the effects of IRS stimulation are consistent with a growing body of literature that collectively suggests that IGF-I-induced differential activation of IRS subtypes is responsible for multiple, distinct biological phenotypes. For example, IGF-I signaling mediated selectively through IRS-1 correlated with enhanced cell

³ D. Yee *et al.*, unpublished observations.

proliferation in MCF-7L cells (15). Ectopic expression of IRS-1 in LNCaP prostate cancer cells (usually IRS-1 deficient) increased cell adhesion and decreased cell motility (32). In contrast, in MDA-231BO cells (predisposed to metastasize to bone), IGF-I stimulation enhanced cell adhesion and motility via increased IRS-2 activation and signaling (21). In still another study, different human neuroblastoma cells with differential IRS subtype expression displayed unique morphologic changes in response to IGF-I stimulation (33): exclusive IRS-2 signaling resulted in enhanced motility and development of neurite-like processes, but signaling via IRS-1 resulted in increased adherence of cells. Overall, all of the above studies additionally suggest that the IRS subtypes regulate different phenotypes in a tissue-dependent manner. Yet, the precise molecular pathways downstream from the IRS molecules are not well understood.

The major implication from our findings is that a potentially effective and specific treatment strategy for malignant pleural mesothelioma could be pursued by using one of the many emerging anti-IGF strategies. Currently, no standard curative treatment exists for patients with malignant pleural mesothelioma. Only a minority of patients, if they meet specific clinical criteria, can undergo aggressive surgical resection (*i.e.*, extrapleural pneumonectomy). Chemotherapy combinations including the multitargeted antifolate pemetrexed (Alimta) recently resulted in a modest but significant median survival benefit (12.1 months *versus* 9.3 months in the control arm, $P = 0.20$) in patients with malignant pleural mesothelioma; this drug consequently received approval from the Food and Drug Administration (5). Nevertheless, there remains ample opportunity and need for alternative, perhaps more efficacious therapies. The results of our study now offer a clear rationale for attempting to disrupt the IGF system in experimental models of malignant pleural mesothelioma.

Several strategies to interrupt IGF1R signaling have been reported, including suppressing IGF production, disrupting the ligand-receptor interaction, down-regulating cell-surface IGF1R expression, or inhibiting IGF1R activation and downstream signaling (34, 35). Recently described small molecule kinase inhibitors of IGF1R (NVP-ADW742 and NVP-AEW541), which demonstrated significant and selective inhibitory activity in preclinical, *in vivo* cancer models, seem to show the most promise (36, 37). Plans are under way to move into clinical trials. The best approach, however, remains to be defined.

In summary, we have now characterized an altered transcript profile of the IGF system in malignant pleural mesothelioma specimens. Furthermore, we determined that critical IGF proteins were expressed by malignant pleural mesothelioma cells and could be activated by IGF-I treatment. Of major importance, we noticed that IGF-I-induced cell proliferation was mediated by IRS-1, whereas IGF-I-induced cell migration was mediated by IRS-2. In the future, the aberrant IGF gene profile of malignant pleural mesothelioma specimens could be used to identify likely responders to anti-IGF strategies that represent a potential therapeutic approach to malignant pleural mesothelioma. Before this goal can be reached, both additional descriptive and functional experiments on a larger sampling of malignant pleural mesothelioma specimens will be required.

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