Insulin Receptor Expression by Human Prostate Cancers

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BACKGROUND. Although recent laboratory and population studies suggest that prostate cancer may be responsive to insulin, there is a gap in knowledge concerning the expression of insulin receptors on benign or malignant prostate tissue.
METHODS. We immunostained 644 cores on tissue microarrays prepared from 29 prostate tissue samples without malignancies, 78 Gleason grade 3 cancers, 21 Gleason grade 4 cancers and 33 Gleason grade 5 cancers with antibodies against the insulin-like growth factor I receptor and the insulin receptor.
RESULTS. We observed immunoreactivity with both antibodies, which implies the presence of hybrid receptors as well as IGF-I receptors and insulin receptors. Insulin receptor staining intensity was significantly ($P < 0.001$) higher on malignant than benign prostate epithelial cells. Analysis of information from public gene expression databases confirmed that co-expression of insulin receptor mRNA and IGF-I receptor mRNA is common in prostate cancer specimens. RT-PCR methods provided evidence for the presence of mRNA for both IR-A and IR-B insulin receptor isoforms.
CONCLUSION. These observations document the presence of insulin receptors on primary human prostate cancers. The findings are relevant not only to ongoing clinical trials of drug candidates that target IGF-I and/or insulin receptors, but also to the hypothesis that obesity-associated hyperinsulinemia mediates the adverse effect of obesity on prostate cancer prognosis.

KEY WORDS: **prostate cancer; insulin receptor; insulin-like growth factor receptor; Gleason grade; metabolic syndrome; obesity**

INTRODUCTION

Roles for insulin-like growth factors in prostate cancer biology are now well-established [1–8]. Recent clinical and laboratory data support the hypothesis that insulin itself also influences the behavior of prostate cancer. For example, we and others have shown that dietary manipulations that raise insulin levels also lead to more aggressive behavior of experimental prostate cancers [9–11]. Furthermore, population studies provide evidence that prostate cancer patients with higher serum levels of insulin or c-peptide (an analyte highly correlated with insulin) are at increased risk of adverse outcome [12,13]. In the Physicians Health Study, we showed that the risk of prostate cancer-specific death among men diagnosed with prostate cancer was 2.7-fold higher (95% CI 1.4–5.2, $P < 0.05$) in men in the top quartile of c-peptide as compared to men in the bottom quartile [13].

Obesity has been identified as an important adverse prognostic factor for prostate cancer [14–16] and therefore the increasing prevalence of obesity threatens to attenuate recent progress in prostate cancer control (reviewed in Ref. [9]). There has been speculation that obesity-associated hyperinsulinemia may explain the relationship between obesity and prostate cancer behavior, and the biology linking obesity to prostate cancer prognosis has become a topic of interest to investigators working in many disciplines.

Most reports of insulin receptor (IR) expression concern tissues that are "classically" insulin-responsive, such as liver, muscle and fat. Relatively little is known about the expression or physiologic role of IRs on tissues that have not conventionally been regarded as insulin responsive, such as prostate or other cancers. There have been reports of expression of insulin-like growth factor I receptors (IGF-IRs) on prostate cancer tissue [4,17,18], but in the context of the evidence supporting the hypothesis that insulin influences prostate cancer behavior, the gap in knowledge concerning the presence or absence of IRs in prostate cancer tissue is conspicuous.

Both IRs and IGF-IRs exist as tetrameric complexes comprised of two half receptors, each of which is comprised of an alpha chain with its corresponding beta chain [19,20]. It is now recognized that in cells that co-express IRs and IGF-IRs, hybrid receptors can form [21–23] (reviewed in Ref. [21]). These consist of a half-insulin receptor (insulin receptor alpha chain + insulin receptor beta chain) complexed with a half IGF-I receptor (IGF-I receptor alpha chain + IGF-I receptor beta chain). Insulin, IGF-I and hybrid receptors all exhibit ligand-dependent tyrosine kinase activity, and activate the AKT and MAPK signalling pathways [19–22]. IGF-IRs can be activated by physiologic concentrations of IGF-I and IGF-II, but not insulin [21]. Classically, IRs bind only insulin, but there now is evidence that there are two insulin receptor isoforms (IR-A and IR-B), and that IR-A can bind IGF-II [21,24,25]. Hybrid receptors bind IGF-I and IGF-II, and their affinity for insulin is an active research topic [21]. There are 6 potential members of the insulin/IGF-I receptor family: IR-A/IR-A, IR-A/IR-B, IR-B/IR-B, IGF-IR/IR-A and IGF-IR/IR-B.

There are important limitations to current detection methods for insulin/IGF-I receptor family members on intact tissue specimens. No antibodies reliably distinguish IR-A from IR-B in conventional immunohistochemistry (IHC): this distinction can only be made at the mRNA level. Antibodies used in IHC are raised against either the alpha chain or the beta chain of the IR or the IGF-IR. Thus IHC can provide evidence for or against presence of a specific component of the tetrameric receptor, but cannot determine if the detected protein is part of a hybrid receptor or of a "pure" insulin or IGF-I receptor. In retrospect, therefore, the reports of IHC evidence for IGF-IR expression in prostate cancer specimens may be re-interpreted as providing evidence for the presence of either IGF-IRs or of hybrid receptors. A demonstration of the absence of IRs on prostate cancer specimens would provide evidence that the previously observed IGF-IR immunoreactivity is due to "pure" IGF-I receptors, while the detection of IRs would suggest that a mixture of IRs, hybrid receptors, and IGF-IRs is present.

Further characterization of insulin/IGF-I receptor family members is of interest not only in the context of the experimental and epidemiologic evidence cited above, but also in the context of the considerable ongoing clinical and laboratory research concerning drug candidates that target insulin and/or IGF signalling by anti-ligand antibody, anti-receptor antibody, or small molecule receptor tyrosine kinase inhibitor strategies [1,26].

MATERIALS AND METHODS

IR and IGF-IR Immunoblotting for Antibody Specificity Studies

PC-3 and HepG2 cells were grown in RMPI-1640 media containing 10% FBS. 10^5 PC-3 and HepG2 cells were mock transfected or transfected with 200 pmol IR siRNA (Dharmacon, Lafayette, CO) or Alexafluor 488 negative control siRNA using an MP-100 MicroPorator (Digital Biotechnology, Ltd., Seoul, Korea). Cells were also resuspended in siRNA transfection buffer without siRNA and microporated as an additional control. Following microporation, cells were transferred to 2 ml RMPI/10% FBS (to give a final siRNA concentration of 100 nM) and incubated for 72 hr at 37°C. Following incubation, the tumor cells were lysed in RIPA buffer and 20 μg of cell lysate was separated on 10% gels by SDS–PAGE, transferred to nitrocellulose membranes and incubated with 1/1000 rabbit anti-human IR beta pAb (Upstate Cell Signaling Solutions, Lake Placid, NY, #07-724) or rabbit anti-human IGF-IR beta pAb C-20 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, #C-20). The proteins were visualized using HRP-linked secondary antibodies and an ECL chemiluminescent detection system from Amersham.

SKBR3 wild-type and IGF-IR overexpressing SKBR3 cells [27] were grown to 80% confluence in RMPI containing 10% FBS with or without 800 μg/ml G418. The cells were washed once in cold PBS then lysed in RIPA buffer on ice for 10 min. Following protein quantification, 50 μg of cell lysate was separated on 10% gels by SDS–PAGE, transferred to nitrocellulose membranes and incubated with 1/1,000 rabbit anti-human IR beta pAb or rabbit anti-human IGF-IR beta pAb and

visualized using HRP-conjugated secondary antibodies and enhanced chemiluminescent detection (Amersham) as indicated.

Gleason Graded Tissue Microarrays (TMAs)

Slides were prepared from formalin-fixed prostatectomy specimens obtained from the Vancouver General Hospital. The patients had no prior treatment. Benign and cancer sites were identified and marked in donor paraffin blocks using matching H&E reference slides. Gleason grade was assigned according to conventional criteria. Two TMAs were constructed using a manual tissue microarrayer (Beecher Instruments, Silver Spring, MD). Each marked block was sampled 4 times with a core diameter of 0.6 mm and arrayed in rectangular pattern with 1 mm between the centers of each core. The total number of patient samples in these TMAs is 161, each in quadruplicate. The TMA paraffin block was sectioned into 0.5 μm sections and mounted on positively charged slides.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed on sequential sections of the Gleason graded TMA with the polyclonal anti-IR beta subunit antibody or the polyclonal IGF-IR beta subunit antibody described above, and visualized by incubation with horseradish peroxidase-conjugated, biotin–streptavidin goat anti-rabbit IgG secondary antibody system and solvent resistant DAB Map kit (DAKO, Inc., Mississauga, ON, Canada) using a Discover XT™ autostainer (Vantana Medical System, Tuscan, AZ). Slides were counterstained with hematoxylin and staining bluing reagent. Nonspecific reactivity was assessed by omission of the primary antibody and comparison to intrinsic stromal immunoreactivity. Specificity of IGF-IR beta subunit immunoreactivity has been previously demonstrated [4].

IHC Scoring Method

Slides were digitally imaged with a BLISS system (Bacus Lab, North Lombard, IL) and staining intensity was visually scored by a pathologist (LF) using a 4 point scale scoring system for which 0 represents no staining by any neoplastic cells, 1 represents faint or focal staining, 2 represents staining of moderate intensity in at least a quarter of neoplastic cells, and 3 represents intense staining by the majority of neoplastic cells.

Survey of Public Gene Expression Databases

Two data sets with appropriately deposited [28] raw data were identified: GSE8218 (http://www.ncbi.nih.gov/geo/query, 2007) (Gene Expression Omnibus) and E-GEOD-3325 (Ref. [29]) (Array Express). As microarray data from tissue samples is of varying quality, we selected only those data set samples that showed good sensitivity (scaling factor<4) and evidence of intact mRNA (one of the two 3′ to 5′ mRNA degradation indicators <4). We used the Unigene version 10 updated probe set definition [30] to provide better precision and accuracy and to overcome interpretation problems related to conflicting id gene references [31]. We evaluated whether IR and IGF-IR mRNAs were present in the data sets of interest using the "present, marginal and absent" calling procedure implemented in the Affymetrix library. Expression levels were calculated using RMA (Hs.465744 for IR and Hs.643120 for IGF-IR).

Detection of Transcripts for Insulin Receptor Isoforms

Laser capture microdissection (LCM) and cDNA preparation. Prostate samples embedded in OCT (Tissue bank, Prostate Centre at Vancouver General Hospital) were used for LCM. In an RNase free environment, 8 μm sections were sectioned by cryostat and mounted onto special membrane slides from P.A.L.M. Micro laser (Technologies, Bernried, Germany). Then they were fixed in 90% cold Ethanol for 10 min. After rapid staining (20 sec) with modified hematoxylin (EM science, Gibbstown, NJ), slides were rinsed with DEPC H_2O and dehydrated in the 70% and 100% for 3 min. Using P.A.L.M. Micro laser system (P.A.L.M. Micro laser Technologies, Bernried, Germany) approximately 10,000 cells were collected per sample. Total RNA (∼150 ng) was then isolated according to the Arcturus Pico Pure kit protocol (Arcturus Bioscience, Inc., Mountain View, CA). In order to make cDNA, total RNA extracts from cells were reverse transcribed using random hexamers (Perkin-Elmer Applied Biosystems, Branchburg, NJ) and 20 units of Moloney murine leukemia virus reverse transcriptase M-MLV (Invitrogen) in 20 μl total volume at 25°C for 10 min and at 37°C for 60 min. Finally, the reaction was stopped by exposure to 95°C for 5 min.

IR mRNA quantitation. RT-PCR of IR-A and IR-B isoform expression was performed on cDNA obtained from six of the microdissected samples and from MCF7 cells as previously described [32] using oligonucleotide primers spanning nucleotides 2,229–2,250 (5′-AACCAGAGTGAGTATGAGGAT-3′) and 2,844–2,865

(5′-CCGTTCCAGAGCGAAGTGCTT-3′) flanking exon 11 of the human insulin receptor sequence to produce DNA fragments of 600 and 636 bp for IR-A and IR-B, respectively, and resolved by agarose gel electrophoresis.

Statistical Analysis of TMA Data

Prior to statistical analysis, data were square-root transformed to satisfy the assumptions of analysis. Statistical significance was evaluated using the GLM Procedure. Two-way analysis of variance was used to determine if a significant difference present between groups. Additionally, least-squares means post hoc for multiple unpairwise comparisons of means (LSMEANS statement with Bonferroni correction) was applied. All statistical analyses were performed using Statistical Analysis System software, version 9.1.3 (SAS Institute, Cary, NC). P values <0.05 were considered significant.

RESULTS

Antibody Specificity

In order to provide evidence to support the hypothesis that the anti-IR and anti-IGF-IR antibodies used in the immunostaining in fact distinguished between the two receptors, lysates from PC-3 and HepG2 cells subjected to siRNA suppression of IR expression were immunoblotted as shown in Figure 1A. IR beta-subunit immunoreactivity was suppressed in lysates of IR siRNA transfected cells (lanes 1 and 4) while no change in IGF-IR beta-subunit immunoreactivity was detected. Furthermore, IGF-IR immunoreactivity was increased in IGF-IR beta-subunit overexpressing SKBR3 cells as compared to parental SKBR3 cells, while no difference in IR beta-subunit immunoreactivity was observed (Fig. 1B). As an extra positive control, we observed the expected IR staining pattern in human placental tissue (Fig. 1C). These results indicate that the anti-IR and anti-IGF-IR antibodies preferentially bind to the appropriate receptor molecule.

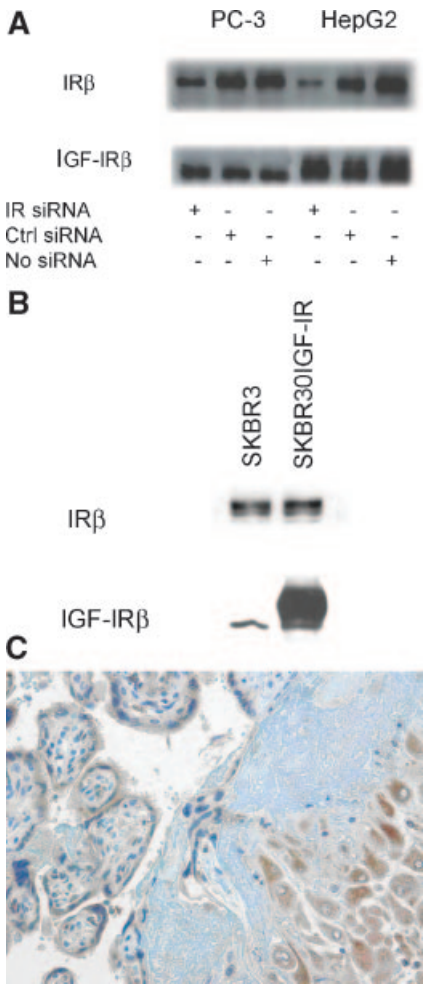

Figure I. Antibody characterization. **A**: PC3 and HepG2 cells were grown in RMPI containing 10% FBS. Cells were trypsinised and transfected with 200 pmol IR siRNA or negative control siRNA and then lysates were immunoblotted as described in Materials and Methods Section. **B**: Lysates from SKBR3 wild-type and IGF-IR overexpressing SKBR3 cells were prepared and immunoblotted as described in Materials and Methods Section. **C**: Human placenta was immunostained with the anti-IR antibody as described in Materials and Methods Section.

Immunohistochemistry

Preliminary visual scoring suggested that IR immunoexpression in prostate cancer was more than two fold higher than that in benign prostatic hyperplasia. The IR-stained tissue shows rather homogenous membranous and cytoplasmic staining in the cancer cells and discontinuous staining in the basal layer cells of the benign glands. Stroma was uniformly scored negative for IR expression. In general, IGF-IR staining is mostly membranous but occasionally cytoplasmic. It is present in the basal cells and luminal cells of the benign glands as well as cancer cells. IGF-IR immunoreactivity was observed sparsely in the stromal compartment, with light staining primarily in smooth muscle cells. Benign prostatic epithelial cells expressed uniform levels of IGF-IR.

Quantitative analysis of IR and IGF-IR staining are provided in Table I, and representative images are shown in Figure 2. These data were obtained from staining scores of 644 cores from 161 prostatectomy specimens for each antibody. Variation of staining scores among quadruplicates of each tumor sample was always $<5\%$ for both immunostains. Overall, mean

TABLE I. Quantitation of Immunohistochemistry Staining Results

Histopathology of specimen	Number of samples	IR staining score, mean SE	IGF-IR staining score, mean SE
A. Quantitation of tissue microarray immunostaining data for IR and IGF-IR performed and quantified as described in Materials and Methods Section			
BPH	29	1.062 0.097	1.908 0.067
Gleason 3	78	1.820 0.052	1.901 0.052
Gleason 4	21	2.373 0.122	1.883 0.091
Gleason 5	33	1.854 0.089	1.955 0.075
Total	161		

	BPH	G3	G4	G5
B. Significance of differences in IR staining scores according to tissue type				
BPH	—			
G3	0.0001	—		
G4	0.0001	0.0032	—	
G5	0.0001	0.05419	0.0155	—

	BPH	G3	G4	G5
C. Significance of differences in IGF-IR staining scores according to tissue type				
BPH	—			
G3	0.3239	—		
G4	0.3688	0.1288	—	
G5	0.0892	0.4488	0.4603	—

For B and C, significances are expressed as *P* value derived from multiple comparisons as described in statistical methods.

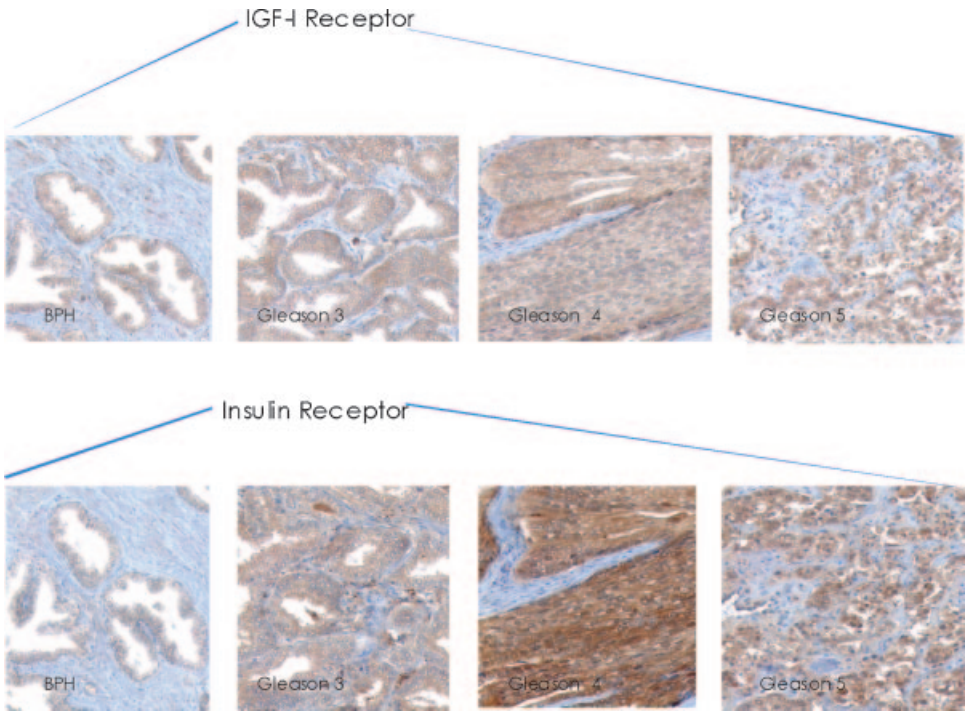

Figure 2. Representative immunostaining of prostate cancer specimens. Six hundred fifty-two tissue microarray cores, representing quadruplicates of 161 benign or malignant prostate tissue samples, were prepared and stained as described in Materials and Methods Section. Representative images are provided, and a summary of quantitative staining data are in Table I.

IGF-IR expression scores were not statistically different by histopathology category (two-way ANOVA $P = 0.9547$). In contrast, IR scoring was increased in neoplasic tissue specimens relative to non-transformed tissue specimens, and this difference was significant (two-way ANOVA $P = 0.0001$), as shown in Table I.

Isoform-Specific Insulin Receptor mRNA Expression in PCa Specimens

In order to confirm expression of IR in PCa specimens, IR mRNA levels were assessed in laser capture microdissected cancer samples by RT-PCR (Fig. 3). Using IR expression in MCF-7 cells as a positive control, five of six PCa samples showed presence of IR mRNA. In all five cases, PCR products consistent with the presence of both IR-A and IR-B insulin receptor isoforms were detected.

Evidence From Public Data Repositories of Insulin Receptor Expression in Prostate Cancer

As mRNA was not available from all prostatic samples, we evaluated evidence for IR mRNA and IGF-IR mRNA expression in publicly available gene expression data sets to determine whether the IR and IGF-IR IHC and PCR results we obtained were consistent with IR mRNA expression profiles measured in independent PCa specimens. Two data sets with appropriately deposited [28] raw data were identified: GSE8218 (http://www.ncbi.nih.gov/geo/query, 2007) (Gene Expression Omnibus) and E-GEOD-3325 (Ref. [29]) (Array Express). Of 75 PCa samples examined from these data sets, 96% showed evidence of significant expression of both IR and IGF-IR mRNA. These results provide further evidence that co-expression of IR and IGF-IR is common in prostate cancer.

Figure 3. IR isoform expression in prostate cancers. As detailed in Materials and Methods Section, RT-PCR was performed after RNA was extracted from laser capture-isolated neoplastic prostate cancer tissue in order to detect the presence or absence of the IR-A and IR-B insulin receptor isoforms. Results from six prostate cancer samples and the MCF-7 cells line, used as a control, are shown.

DISCUSSION

In the case of both IGF-IRs and IRs, the component alpha and beta chains are derived by proteolytic processing of a single protein translated from a single gene, so the presence of a beta chain is associated with the presence of the related alpha chain, which associate to form a "half-receptor." These "half-receptors" combine to form mature tetrameric receptors. Binding of two homologous "half-receptor" yields classic IGF-I or insulin receptors, while heterogenous "half-receptor" combinations form "hybrid receptors." Our results demonstrate that it is common for both insulin receptor beta chain immunoreactivity and IGF-I receptor beta chain immunoreactivity to be present on prostate cancer tissue. Therefore, our major conclusion is that prostate cancers display a mixture of IGF-IRs, IRs, and hybrid receptors on their surface.

We considered the possibility that our data might be spurious due to cross reactivity of the anti-insulin receptor beta chain antibody with the IGF-I receptor beta chain, but the technical control data presented argue against this possibility. Furthermore, we showed that public gene expression data bases provide readily available evidence for presence of both IR and IGF-IR mRNA in prostate cancer specimens, making our interpretation of the IHC results plausible. We anticipate that the results reported here may be extended to other common epithelial tumors, such as breast, lung and colon cancer, based on similar evidence for IR mRNA expression in these tissues (data not shown).

Our finding that insulin receptor immunoreactivity is increased in transformed prostatic epithelium deserves further study. It raises the possibility that higher grade cancers may be more sensitive to stimulation by insulin than lower grade cancers. This implication is based on the concept that if there is a surplus of insulin half receptors relative to IGF-I half-receptors, there will be a higher probability of association of two insulin half-receptors to form a pure IR (which is responsive to physiologic insulin concentrations) than to form a hybrid receptor (which is less responsive to insulin). However, this remains speculative at present because it is not possible to be rigorously quantitative in interpreting differing levels immunoreactivity of two different proteins. However, while the relative level of IRs, hybrid receptors, and IGF-IRs is unclear, the evidence that insulin receptor beta chain immunoreactivity is higher in transformed than benign prostate epithelial cells and varies with Gleason grade is strong.

Emerging evidence that hyperinsulinemia predicts poor *prognosis* for prostate cancer patients is in contrast to most (but not all [33,34]) studies that find no relationship or even an inverse relationship between insulin levels and *risk* of prostate cancer (reviewed in

Ref. [35]). This dichotomy could reflect biological reality if advanced, high grade cancers are more insulin sensitive than untransformed epithelial cells or low-grade lesions, which is compatible with our observations concerning insulin receptor levels. However, the relationship between risk factors and prognostic factors for prostate cancer is complex and may vary with PSA screening and medical care systems: in some settings a "case" may only be detected if there has been considerable disease progression. In this setting, the proportion of cases with poor prognosis and high grade may be high, in contrast to screened populations where more cases are detected, but many are of good prognosis and low grade.

Prior work (reviewed in Ref. [18]) provided consistent evidence that IGF-IRs are expressed in prostate cancer [4,17,18] but the relationship of IGF-IR level to grade has been the subject of controversy. Our study examined substantially more specimens than those described in prior reports and confirmed the presence of IGF-IR in prostate cancer, but did not detect an increase in IGF-IR immunostaining intensity with grade. There is now experimental evidence that IGF-IR level may vary according to androgen dependence [4,5] and be influenced by androgens [3,4,36] and estrogens [37]. Therefore, future research to resolve the controversy may need to simultaneously take into account both grade and androgen dependence. It will be important in future work to extend these studies to the relationship of androgen independence to insulin receptor levels. Such future studies will require immunohistochemical methods, but in view of the limited evidence we report here concerning expression of mRNAs for both the IR-A and IR-B isoforms of the insulin receptor, it will also be of interest to determine if relative abundance of these isoforms varies with grade or androgen dependence.

CONCLUSION

We have demonstrated that expression of insulin receptors is common on primary human prostate cancer specimens. In the context of our recent observation that diet-induced hyperinsulinemia is associated with increased AKT activation and aggressive tumor behavior in a prostate cancer model [9], the results reported here provide further evidence consistent with the hypothesis that prostate cancers are influenced by insulin. In particular, we propose that in obese cancer patients (or cancer patients who are normal weight but "metabolically obese" [38]), the insulin resistance present in classic insulin-responsive organs such as liver, fat, and muscle [39] leads to hyperinsulinemia, which may lead to aggressive behavior of those cancers that are IR positive and insulin sensitive. Finally, in the context of data showing that castration therapy is associated with hyperinsulinemia [40], our demonstration of insulin receptors in prostate cancer tissues motivates research concerning roles of insulin signalling in progression of prostate cancer to castration-resistant behavior. The possibility that metformin [41,42] or targeted therapies [1,43] against insulin—IGF signalling will be useful in prostate cancer treatment is under investigation.

ACKNOWLEDGMENTS

This work was supported by grants to MP and MEC from the National Cancer Institute of Canada (NCIC), by a grant to MP from the Prostate Cancer Foundation (PCF), and by grants to MP and MEG from the Canadian Prostate Cancer BioResearch Network (CPC-BioNet). OL is supported by a postdoctoral fellowship from the Swedish Research Council. EP is supported by a research fellowship from the Israel Cancer Research Fund (ICRF).

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