Insulin-like Growth Factor-1 Receptor in Uveal Melanoma: A Predictor for Metastatic Disease and a Potential Therapeutic Target

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Purpose. To investigate the expression of the insulin-like growth factor-1 receptor (IGF-1R) with special focus on its role in cell growth in uveal melanoma.

Methods. Paraffin material from 36 clinicopathologically well characterized cases of primary uveal melanomas (18 of which had metastasized to the liver) with more than 15 years' follow-up was used for immunohistochemical analysis. In the experimental studies, three uveal melanoma cell lines (OCM-1, OCM-3, and 92-1) were used. The expression level of IGF-1R in the cell lines was modulated by glycosylation inhibitors, and the IGF-1R was neutralized with the antibody αIR-3. Expression of IGF-1R was assayed by Western blot analysis and immunohistochemistry. Cell growth and survival were analyzed by cell counting, thymidine incorporation, and viability assays.

Results. Western blot analysis and immunohistochemistry confirmed that IGF-1R is expressed in uveal melanoma. Although 10 of 18 patients who died of metastasizing disease showed high IGF-1R expression, only 5 of 18 tumors from patients who survived for 15 years or more after enucleation exhibited a high IGF-1R expression. Kaplan-Meier analysis showed a significant association ($P = 0.035$) between a high IGF-1R expression and death due to metastatic uveal melanoma. Using in vitro experimental models, we found that inhibition of the IGF-1R activity (tyrosine phosphorylation) was associated with a drastic decrease in uveal melanoma cell viability.

Conclusions. These data suggest an important role of IGF-1R in uveal melanoma. The significant association between high IGF-1R expression and death due to metastatic disease may be explained by the fact that IGF-1 is mainly produced in the liver, which is the preferential site for uveal melanoma metastases. These data also point to the possibility of therapeutically interfering with IGF-1R, which appears to be expressed preferentially in uveal melanomas that appear to follow an aggressive clinical course. (*Invest Ophthalmol Vis Sci.* 2002;43:1–8)

Uveal melanoma is the most common primary intraocular malignant tumor in adults, with an annual incidence of six cases per million in whites.[1] It has a high mortality rate due to a high incidence of metastases, which have a strong preference for the liver.[2,3] Because chemotherapy against metastatic uveal melanoma has not as yet been shown effective, new molecular targets have to be established to design appropriate pharmacologic agents to provide a new treatment modality.

That uveal melanoma shows early and preferential dissemination to the liver raises the possibility that hepatic environmental factors are important for the growth, survival, and progression of this malignancy. Such favorable conditions may involve growth factors produced in the liver—for example, hepatocyte growth factor (HGF; scatter factor). HGF exerts its biological effects through binding to the plasma membrane receptor c-Met. An interesting study by Hendrix et al.[4] has shown that uveal melanoma cells with metastatic phenotype express c-Met.

Another growth factor mainly produced in the liver is insulin-like growth factor (IGF)-1, which binds to the IGF-1 receptor (IGF-1R), which is a heterotetrameric plasma membrane glycoprotein composed of two α-subunits (130 kDa each) and two β-subunits (96 kDa each) linked by disulfide bonds.[5,6] Ligand binding to the IGF-1R induces activation of the intrinsic tyrosine kinase of the β-subunit, leading to phosphorylation of IGF-1R and several intracellular proteins, including the family of insulin receptor substrates (IRSs)—for example, IRS-1.[7] This leads to activation of Ras, mitogen-activated protein kinase, and phosphoinositol-3-kinase (i.e., events involved in the mitogenic cascade).[5] In an increasing number of malignant cell types, IGF-1R has been shown essential for tumorigenesis as well as for the establishment and maintenance of a transformed phenotype.[5,7] Furthermore, protection from apoptotic cell death has been ascribed to IGF-1R.[5,7]

Previously, we have shown that N-linked glycosylation is critical for the translocation of IGF-1R from the endoplasmic reticulum to the cell surface.[8–11] Treatment with tunicamycin (TM), which is a specific inhibitor of N-linked glycosylation, or lovastatin, which is a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, both downregulate IGF-1R at the cell surface.[8–11] The inhibitory effect of lovastatin on N-linked glycosylation is explained by a decreased synthesis of dolichyl phosphate, which acts as a carrier of oligosaccharides in the assembly of glycoproteins.[12] Growth arrest and apoptosis follows the decreased cell surface expression of IGF-1R.[10,13,14]

The purpose of this study was to evaluate the expression of IGF-1R in uveal melanoma regarding its correlation with clinical behavior and regarding its role in growth and survival of uveal melanoma cells.

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Table 1. Clinicopathologic Parameters

Tumor No.	Sex/Age (y)	IGF-1R*	Tumor Size (mm)	Tumor Location†	Cell Type‡	Tumor Invasion§	Follow-up (mo)	Survival‖
1	M/67	0	10	1	2	1	186	2
2	M/55	2	14	1	4	1	214	2
3	F/59	1	8	2	4	1	242	0
4	M/48	1	16	2	1	1	242	0
5	M/72	1	11	2	2	1	203	2
6	M/59	1	15	2	1	1	245	0
7	M/78	2	14	1	4	1	243	0
8	F/62	1	7	1	4	1	231	0
9	F/44	0	15	2	4	2	238	0
10	M/54	1	10	1	2	2	236	0
11	M/56	2	8	1	4	2	14	1
12	M/65	2	9	3	4	1	57	1
13	F/57	1	6	2	1	1	224	0
14	F/72	1	9	1	1	1	224	0
15	F/70	1	9	2	1	2	35	1
16	F/60	1	6	1	1	1	219	0
17	M/55	2	11	1	2	1	58	1
18	M/60	1	7	1	1	1	218	0
19	F/61	2	12	2	4	1	225	0
20	M/23	2	13	2	2	2	216	0
21	F/63	1	15	1	1	1	150	1
22	M/69	2	12	1	1	1	50	1
23	F/54	1	10	1	1	1	75	1
24	M/33	1	9	1	1	1	202	0
25	F/47	2	8	1	3	2	201	0
26	F/69	2	7	2	3	1	32	1
27	M/83	2	16	2	1	2	33	1
28	M/75	2	13	2	3	1	1	1
29	M/44	1	12	2	1	1	81	1
30	M/61	1	11	1	1	1	121	1
31	M/66	1	15	3	2	1	107	1
32	M/64	1	17	2	2	2	52	1
33	M/66	2	15	3	2	2	51	1
34	F/77	1	13	1	3	3	1	1
35	M/87	2	15	1	4	2	21	1
36	F/49	2	12	2	2	1	29	1

* Expression: 0, <15% of cells stained; 1, 15%–50% of cells stained; 2, >50% of cells stained.
† 1, Posterior choroid; 2, anterior choroid; 3, ciliary body.
‡ 1, Spindle; 2, mixed; 3, epitheloid; 4, necrotic.
§ 1, <⅓ of sclera; 2, >⅓ of sclera but no sign of extrascleral invasion; 3, extrascleral invasion.
‖ 0, Still alive; 1, death due to tumor; 2, dead of other cause.

Materials and Methods

Chemicals

Rabbit antisera against IGF-1R (N-20) and anti-phosphotyrosine (PY99) were from Santa Cruz Biotechnology (Santa Cruz, CA), and the macrophage marker CD68 (clone KP1 #M0814) was from Dako (Santa Barbara, CA). A mouse monoclonal antibody directed to the human IGF-1R (αIR-3) was purchased from Oncogene Science (Manhasset, NY). Lovastatin was obtained from Merck, Sharp & Dohme (Rahway, NJ) and was converted to its sodium salt by incubation in 0.1 M NaOH at 50°C for 2 hours. All other chemicals, unless stated otherwise, were from Sigma (St. Louis, MO).

Tumor Material

Paraffin blocks from 36 patients (14 females and 22 males), derived from surgically removed primary uveal melanomas, were used for immunohistochemistry (IHC; Table 1). The ages of the patients varied between 23 and 87 years with a mean age of 61 years at diagnosis. Eighteen patients died of tumor-related causes, 3 died of other causes, and 15 were still alive at the time of follow-up. The follow-up time was 15 years or more (Table 1).

Fresh-frozen uveal melanoma tissue samples from 11 patients were used for confirmatory Western blot analysis. All parts of the study were conducted in compliance with the Declaration of Helsinki.

Cell Culture

Three cell lines obtained from human primary uveal melanomas (OCM-1, OCM-3, and 92-1) were used. OCM-1 and OCM-3 were kindly provided by June Kan-Mitchell (Wayne State University, Detroit, MI). Cell line 92-1 was established in one of our laboratories (Leiden University Medical Center, Leiden, The Netherlands). The OCM-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with10% fetal bovine serum (FBS), and the OCM-3 and 92-1 cells in RPMI 1640 supplemented with 10% FBS and 3 mM L-glutamine.

Cells were grown in monolayers in tissue culture flasks, maintained in a 95% air-5% CO_2 atmosphere at 37°C in a humidified incubator. For experimental purposes, the cells were cultured in 35- or 60-mm plastic Petri dishes. Cells were seeded at a density of 3000 to 5000 cells/cm^2, and experiments were initiated when cells had reached subconfluence.

Immunoprecipitation and Western Blot Analysis

Preparation of cell membranes was performed essentially as described elsewhere.[15] In brief, cells were harvested and homogenized in a buffer containing 0.32 M sucrose, 1 mM taurodeoxycholic acid, 2 mM $MgCl_2$, 1 mM EDTA, 25 mM benzamidine, 1 μg/mL bacitracin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinine, 10 μg/mL soybean trypsin inhibitor, and 10 μg/mL leupeptin. After a 10-minute

centrifugation at 600*g* (4°C) the pellet (containing unbroken cells, nuclei, and cytoskeleton) was discarded. The supernatant was then centrifuged at 17,300*g* for 30 minutes. The resultant pellet, containing cell membranes, was used for Western blot analysis.

For immunoprecipitation, cell lysates were obtained using PBSTDS buffer (100 mL 1× PBS with 10 mL 100% Triton X-100, 5 g sodium deoxycholate, and 1 g SDS in 100 mL deionized water), containing the aforementioned protease inhibitors. An equal amount of protein from each sample was immunoprecipitated with 15 μL protein G plus-agarose and 1 μg αIR-3. After a 24-hour incubation at 4°C on a rocking platform, the immunoprecipitates were collected by centrifugation in a microcentrifuge at 2500 rpm for 15 minutes. The supernatant was discarded, and the pellet was washed four times with 1 mL PBSTDS. The material was then dissolved in sample buffer for SDS-PAGE. Protein samples (from plasma membrane preparations or total cell lysates) were dissolved in a sample buffer containing 0.0625 M Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, bromophenol blue, and dithiothreitol. Sample amounts obtained from 100 μg cell protein were analyzed by SDS-PAGE with a 4% stacking gel and a 10% separation gel, essentially according to the protocol of Laemmli.[16] Molecular weight markers (Bio-Rad, Richmond, CA) were run simultaneously. For Western blot analysis, the proteins were transferred overnight to nitrocellulose membranes (Amersham, Buckinghamshire, UK) and then blocked for 1 hour at room temperature in a solution of 5% (wt/vol) skimmed milk powder and 0.02% (wt/vol) Tween 20 or 1% (wt/vol) skimmed milk powder and 1% bovine serum albumin (immunoprecipitation) in PBS (pH 7.5). Incubation with the primary antibody, against the α-subunit of IGF-1R (N-20), was performed for 1 hour at room temperature, followed by three washes with PBS and incubation with a biotinylated secondary antibody (Amersham) for 1 hour. After a 15-minute incubation with streptavidin-labeled horse peroxidase, detection was performed using enhanced chemiluminescence with autoradiographic film (Hyperfilm-ECL; Amersham)

Cell Growth Assay

To determine DNA synthesis, cells were cultured in 35-mm dishes and, after the experimental conditions, labeled with [3H]thymidine (1 μCi/mL, 5 Ci/mmol) for 4 hours. The acid-precipitable material was then taken for scintillation counting as described elsewhere.[8] Cell proliferation was measured by determining the number of cells attached to the plastic surface of duplicate 35-mm dishes. This was performed by microscopic counting of all cells in ink-marked areas on the dish bottom. By repeating the counting after specified time intervals, changes in the number of attached cells could be observed.[10]

Cell Viability Assay

A cell proliferation kit (Kit II) was purchased from Roche Diagnostic GmbH (Mannheim, Germany). The test is based on a colorimetric change of the yellow tetrazolium salt XTT into orange formazan dye by the respiratory chain of viable cells.[17] Cells seeded at a concentration of 5000/well in 100 μL medium in a 96-well plate were treated with different drugs in specified concentrations. After 24 hours, cells were incubated according to the manufacturer's protocol with the XTT labeling mixture. After 4 hours, the formazan dye was quantified using a scanning multiwell spectrophotometer with a 495-nm filter. The absorbance is directly correlated with the number of viable cells. To draw the standard absorbance curve, we used untreated cells seeded at concentrations from 1,000 to 10,000 cells/well with an increasing rate of 1,000 cells/well. All standards and experiments were performed in triplicate.

Immunohistochemistry

Immunostaining was performed using the standard avidin-biotin complex technique (ABC Elite Standard Kit, cat. PK-6100; Vector Laboratories, Burlingame, CA). Deparaffinized, rehydrated sections (two adjacent sections for each specimen) were pretreated with microwaves for 10 minutes in 0.1 M citrate buffer at pH 6.0 (IGF-1R) or digested with 0.05% pronase for 15 minutes (CD68). Before immunostaining, the endogenous peroxidase activity was blocked by hydrogen peroxidase dissolved in methanol (3% hydrogen peroxide in methanol, 1:5 volume) for 30 minutes. Sections were then rinsed in and incubated with blocking serum (1% bovine serum albumin) for 20 minutes followed by incubation with the primary antibodies diluted 1:1000 (IGF-1R) and 1:50 (CD68). The incubation was performed overnight at 4°C. A biotinylated anti-rabbit or anti-mouse IgG was used as a secondary antibody and followed by the ABC complex. The peroxide reaction was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.6 mg/mL with 0.03% hydrogen peroxide) for 6 minutes. Counterstaining was performed by Mayer's hematoxylin. Tris-buffered saline (pH 7.6) was used for rinsing between the different steps. Placenta tissue was used as the positive control for the polyclonal antibody against IGF-1R. In addition, a control peptide against N-20 (SC712P) was used to secure the staining results. All the slides were coded and analyzed in a blinded fashion.

After tissue processing, all cells displaying distinct immunoreactivity were considered positive, irrespective of staining intensity. We classified the results of IGF-1R stainings as zero when less than 15%, low when 15% to 50%, and high when more than 50% of melanoma cells were positive according to an arbitrary scale: 0, negative; 1, low expression; and 2, high expression. At a later stage, and without knowledge of the initial result, the same observer repeated the assessment for each of the 36 uveal melanomas. These specimens were also assessed by an independent observer using the same grading system. The interobserver reproducibility using the κ test was 0.75 (95% confidence interval [CI]0.54–0.95) and $\kappa = 0.95$ for intraobserver reproducibility (95% CI 0.85–1.05). Both observers were masked to results from earlier assessments and to survival data.

Determination of Protein Content

Protein content of cell lysates was determined by a dye-binding assay[18] with a reagent purchased from Bio-Rad. Bovine serum albumin was used as a standard.

Statistical Analysis

Survival data without loss to follow-up were obtained, according to the tenets of the Declaration of Helsinki, for all patients with uveal melanoma from the Swedish National Causes of Death Registry. Time from the date of surgery to death or the end of 1996 was considered censored if the patient was alive at the end of 1996 or had died of any other than a melanoma-related cause. The log-rank test was used to assess survival differences. Calculations were computer based (Statistica; StatSoft, Tulsa, OK; and MedCalc; MedCalc Software, Mariakerke, Belgium).

Results

Studies of Primary Uveal Melanomas

First, we investigated the expression of IGF-1R on 11 fresh-frozen samples from primary uveal melanomas by Western blot analysis, using an antibody specific for the 130-kDa α-subunit of IGF-1R.[8] Eight of the cases resulted in, to a variable extent, a positive signal (Fig. 1). Thus, in three of the uveal melanoma

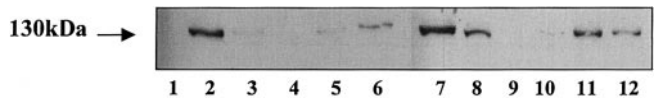


Figure 1. IGF-1R expression in fresh-frozen samples from primary uveal melanoma. Plasma membrane proteins, isolated from normal human fibroblasts (*lane 1*) and 11 cases of primary uveal melanomas (*lanes 2–12*), were subjected to Western blot analysis using an antibody to the α-subunit (130 kDa) of IGF-1R. The same amount of protein was loaded for each case.

cases as well as in normal human fibroblasts (Fig. 1, lane 1), there was no detectable expression of IGF-1R. These 11 samples were obtained from recent surgical cases (follow-up, <2 years) and could not be used for association with clinical outcome. Therefore, we investigated the expression of IGF-1R by IHC on paraffin-embedded surgical specimens of 36 cases of primary uveal melanoma with long follow-up. Positive immunoreactivity was found in 34 of the cases. The expression level regarding intensity of immunoreactivity was scored: no expression, low expression, and high expression, as described in Materials and Methods. An additional immunostaining using the macrophage marker CD68 was also performed on adjacent sections from all specimens. Macrophages usually represented less than 10% of cells within the core of the tumors. Figure 2A shows photomicrographs of immunostaining for IGF-1R and CD68 in a case of uveal melanoma.

Using Kaplan-Meier statistical analysis (Fig. 2B) we found a significant association between high IGF-1R expression and death due to uveal melanoma ($P = 0.035$). In particular, the cases with high IGF-1R expression showed a remarkably fast decrease in survival and the bottom level of 33% was reached as soon as 55 months after enucleation. The corresponding values for the combined cases with no or low IGF-1R expression was 63% at 150 months. In Figure 2C, the association between tumor size (tumor diameter <10 mm versus tumor diameter ≥10 mm), which is an established prognostic parameter for uveal melanoma,[3] and survival is shown. There was a clear trend for decreased survival in tumors of 10 mm or more, but this did not reach statistical significance in this study sample ($P = 0.157$).

In Vitro Assays

In the second part of the study, we investigated the expression of IGF-1R in the three uveal melanoma cell lines OCM-1, OCM-3, and 92-1 and sought to determine how modulation of IGF-1R expression affects cell growth and survival. Each cell line was treated with the IGF-1R blocking antibody αIR-3 (1 μg/mL), which has been shown to be specific for IGF-1R,[19] or with lovastatin (10 μM) or TM (5 μg/mL) for 4 hours. None of these agents caused any decrease in cell viability after 4 hours (data not shown). The choice of the concentrations of TM, lovastatin, and αIR-3 in this study was based on previous dose–response experiments (data not shown). In Figure 3, we show that the basal level of IGF-1R expression at the cell surface, as detected by Western blot analysis of the α-subunit, was almost similar in the three cell lines. As expected from previous studies on other cell types,[8–11] the expression of IGF-1R at the cell surface was decreased after treatment with lovastatin and TM in all cell lines, but not after prior treatment with the monoclonal antibody αIR-3. Consequently, lovastatin and TM reduced ligand-mediated activation of the 96-kDa β-subunit of IGF-1R in all three uveal melanoma cell lines, whereas αIR-3 produced a strong effect only in OCM-1 and OCM-3 cells (Fig. 3). The 92-1 cells responded to treatment with αIR-3 with a moderate decrease in IGF-1R phosphorylation. Differences in modulating IGF-1R phosphorylation using a blocking monoclonal antibody could be explained by different concentrations of the receptor at the cell surface. However, the concentration of αIR-3 used was found to be optimal. Therefore, we believe that αIR-3 binds to all cell surface receptors, but for some other reason it does not block β-subunit phosphorylation completely in the 92-1 cell line. Although αIR-3 mostly has been reported to block IGF-1R activation, exceptions have been demonstrated.[20,21]

In Figure 4, the effects of lovastatin, TM, and αIR-3 treatments on cell and DNA replication and cell survival are demonstrated. As shown in Figure 4A, the basal proliferative rate differed between the three uveal melanoma cell lines. During a 72-hour period, the OCM-1 control cells had increased fivefold in cell number, whereas 92-1 and OCM-3 had increased only 2- to 2.5-fold. All cell lines responded quickly to incubation with αIR-3, lovastatin, or TM (Fig. 4A) and after 24 to 72 hours cell numbers decreased. Consistent with the lower inhibitory effect of αIR-3 phosphorylation of 92-1, the decrease in 92-1 cell proliferation was also less after αIR-3 incubation than with lovastatin and TM treatments. A 24-hour treatment with αIR-3 led to a 60% decrease in DNA synthesis in this cell line (Fig. 4B), whereas cell viability was only slightly (15%) reduced (Fig. 4C). Regarding OCM-1 cells, a drastic reduction of total cell number was observed after TM treatment (Fig. 4A). This was correlated with a drastic decrease in both DNA synthesis and cell viability. Whereas cell replication of OCM-1 cells was efficiently blocked by lovastatin and αIR-3, there was only a moderate or no, respectively, loss of cells (Fig. 4A). However, using the viability assay, it was confirmed that almost all OCM-1 cells had died after the treatment with these two agents (Fig. 4C).

As shown in Figure 4A, TM, lovastatin and αIR-3 reduced the number of OCM-3 cells with similar kinetics, and after 72 hours there was a total cell loss. Although the cell number was decreased by only 20% to 25% after a 24-hour treatment, the OCM-3 cell viability was reduced by as much as 75% to 85% (Fig. 4C). The decrease in DNA synthesis differed somewhat between the three agents. αIR-3 caused a 40% decrease, compared with 70% to 80% for TM and lovastatin.

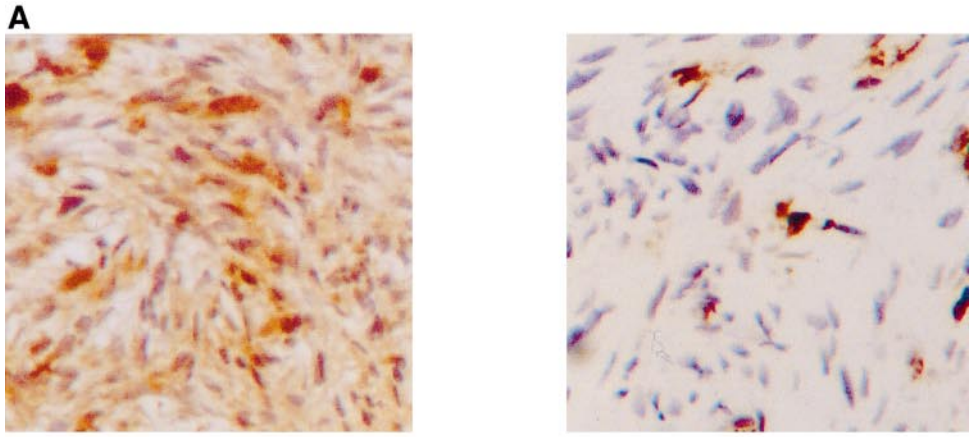
In Figure 5 the level of tyrosine phosphorylation at 4 hours is correlated with DNA synthesis and cell viability. The values are obtained from Figures 3 and 4. As can be seen, there is an especially strong correlation between IGF-1R phosphorylation and cell viability.

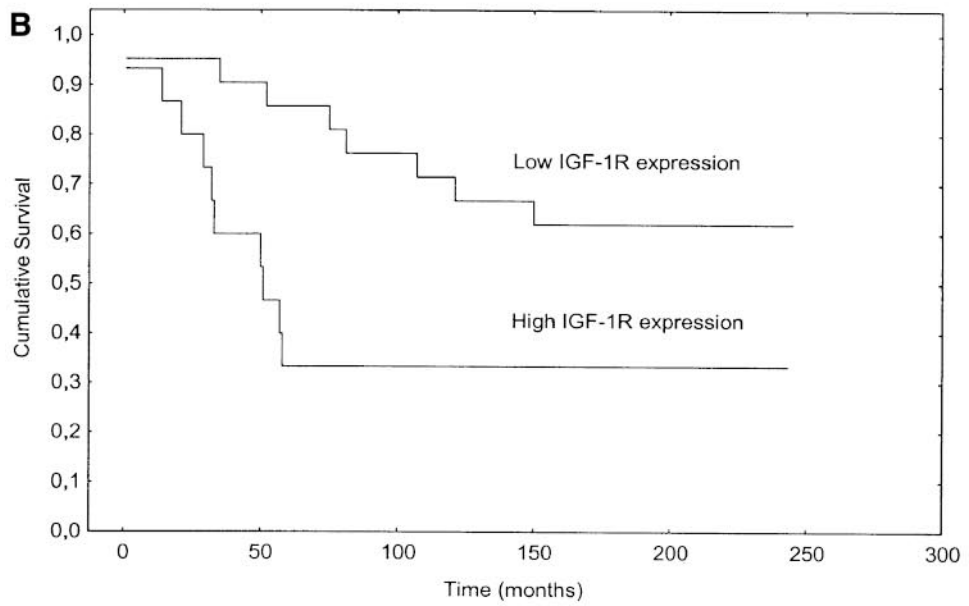
Discussion

The roles of growth factors and growth factor receptors in the regulation of uveal melanoma growth and progression are largely unknown. Ma and Niederkorn[22] found that epidermal growth factor receptor (EGFR) correlated with an increased capacity to metastasize to the liver in nude mice models. This was subsequently confirmed but also was reputed to be the case in humans.[23,24] Studies of uveal melanoma cell lines have shown that transforming growth factor (TGF)-β2 increases tissue-specific plasminogen activator, suggesting a role for this growth factor in metastasis of uveal melanoma.[25]

In several cell types, IGF-1R has been shown to play a pivotal role in cell cycle regulation, differentiation, apoptosis protection, and cell transformation.[7,26–33] Furthermore, a high expression has been shown to be correlated with a poor clinical outcome in certain cancers. Xie et al.[34] recently demonstrated that metastatic dissemination of synovial sarcoma, a highly malignant soft tissue tumor, was significantly correlated with a high expression of IGF-1R in the localized tumor, as assayed by both RT-PCR and Western blot analysis. Egan et al.[35] have reported the importance of IGF-1 and its serum binding protein, IGFBP-3, both of which were found to independently predict metastasis in choroidal melanoma. However, as has been concluded by Baserga,[36] IGF-1 (or IGF-2) can stimulate the growth of tumors, but it is the receptor that defines the outcome and the presence of an IGF-1R is obligatory for malignant transformation.[36] To our knowledge, the present study is the first one to investigate the expression and role of IGF-1R in uveal melanoma.

From the present study we can conclude that IGF-1R is variably expressed in primary uveal melanoma and that (based on our selected sample) a high expression of IGF-1R is associated with a decreased survival in this disease. Although it is





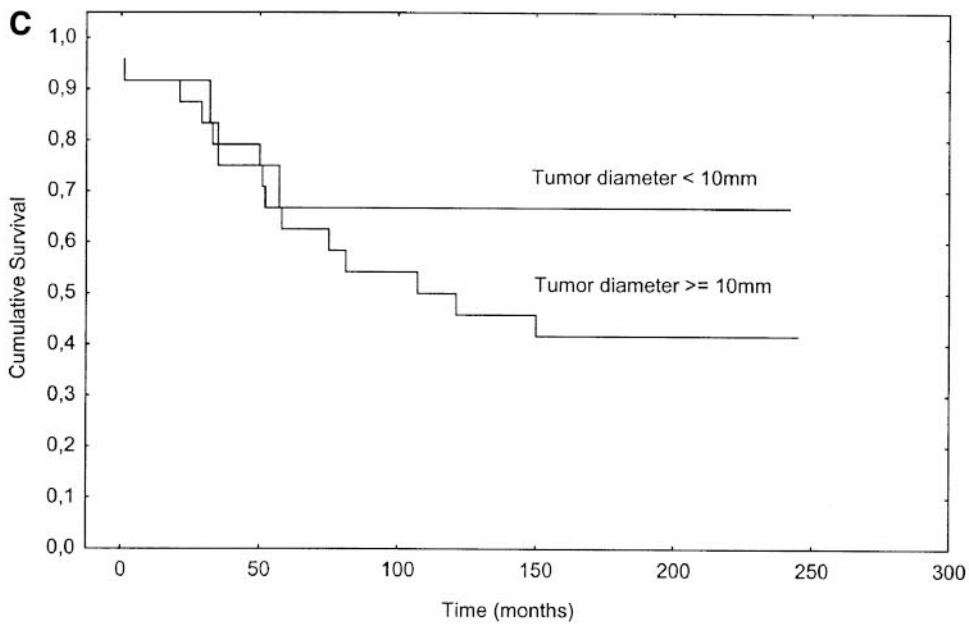


FIGURE 2. (**A**) Photomicrographs of immunostaining for IGF-1R (*left*) and CD68 (*right*) in a case of uveal melanoma. (**B**) Association between IGF-1R expression and death caused by the disease. The 36 cases of uveal melanoma were subdivided into two groups based on IGF-1R expression: no or low expression (21 cases) versus high expression (15 cases). (**C**) The same cases were subdivided into two groups regarding size: tumor diameter less than 10 mm (12 cases) and 10 mm or more (24 cases). Kaplan-Meier analyses were performed.

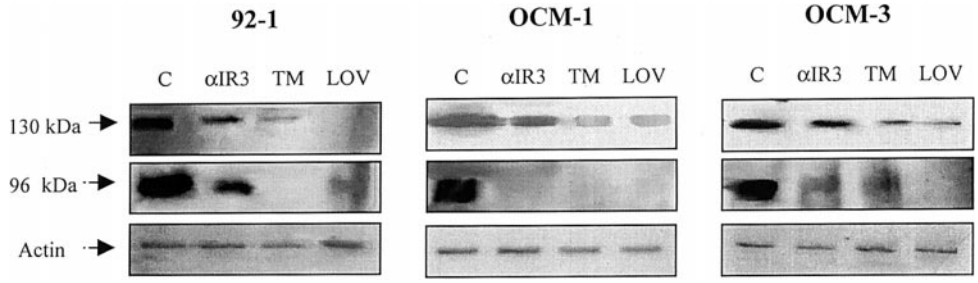


FIGURE 3. Expression and activity of IGF-1R in uveal melanoma cells. OCM-1, OCM-3, and 92-1 cells were treated with αIR-3 (1.0 μg/mL), TM (5 μg/mL), or lovastatin (10 μM) for 4 hours, whereupon proteins were isolated for analysis of expression of the IGF-1R α-subunit at the cell surface (*top*), the tyrosine-phosphorylated IGF-1R β-subunit (*middle*), and expression of actin (loading control; *bottom*). Control cells (C) were untreated.

possible that some macrophages showed positive IGF-1R immunostaining, such macrophages were usually confined to the peripheral part of the tumor. Furthermore, most macrophages could be detected by their morphologic appearance. Our study was exploratory, and the patients were selected to represent one of two extremes (i.e., those who died of uveal melanoma or those who survived 15 years or more). In this respect, our data may be biased and would need confirmation by a larger study of consecutive patients, by means of multivariate analysis.

From our study we can also conclude that growth and survival of uveal melanoma cell lines are strongly dependent on IGF-1R expression and activation. Specifically, we have shown that inhibition of N-linked glycosylation induced by treatment with TM and lovastatin caused decreased IGF-1R expression and consequently decreased tyrosine phosphorylation. Subse-

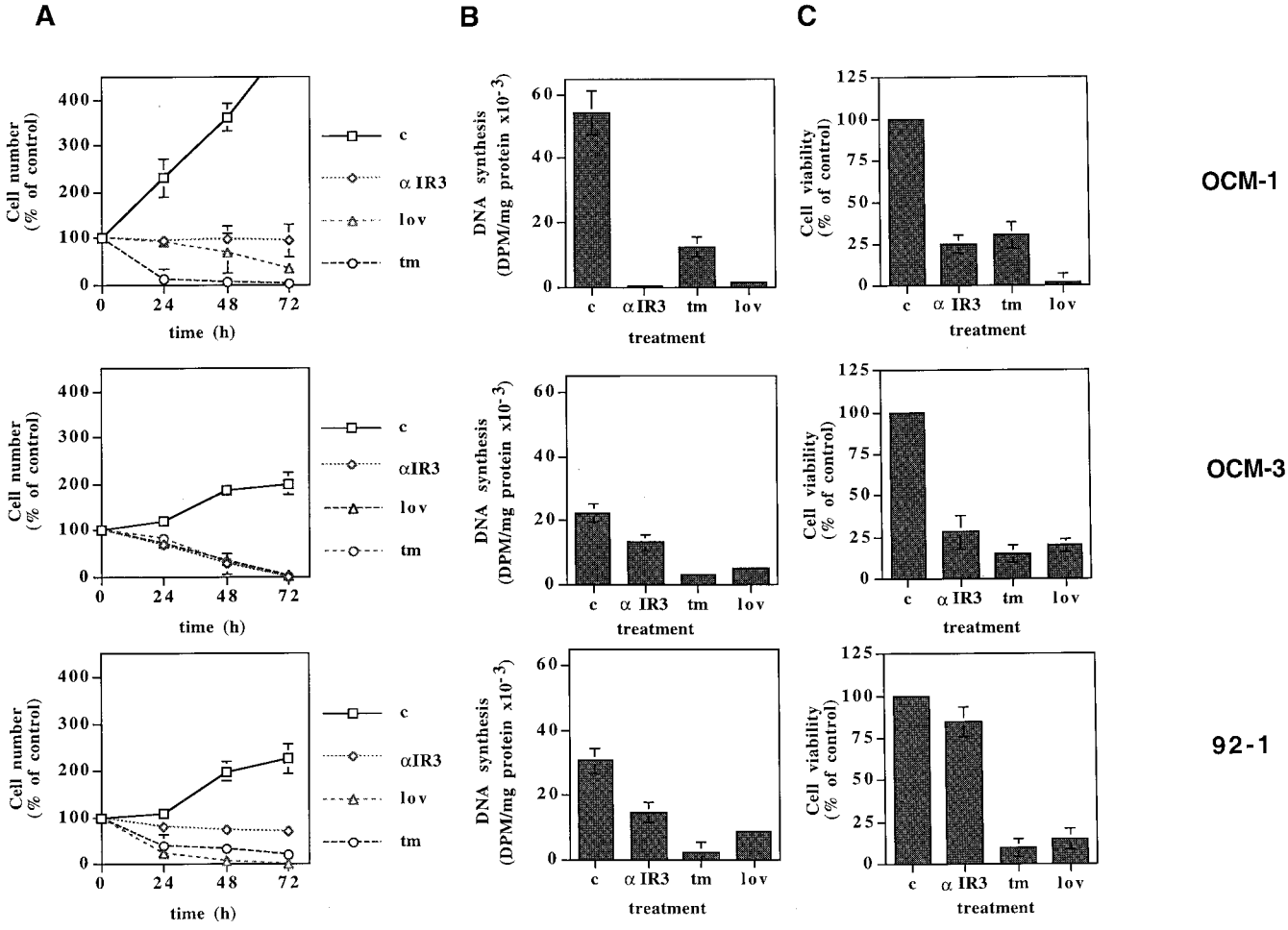


FIGURE 4. Effect of inhibition of IGF-1R on cell number (**A**), DNA replication (**B**), and cell viability (**C**). OCM-1, OCM-3, and 92-1 cells were treated with αIR-3 (1.0 μg/mL), TM (5 μg/mL), or lovastatin (lov; 10 μM). Controls (c) were untreated. Changes in cell number were assayed every 24 hours during a 3-day period (**A**). DNA synthesis (assayed by [3H]thymidine incorporation) and cell viability were analyzed after a 24-hour period. Mean values and SD of duplicates are indicated.

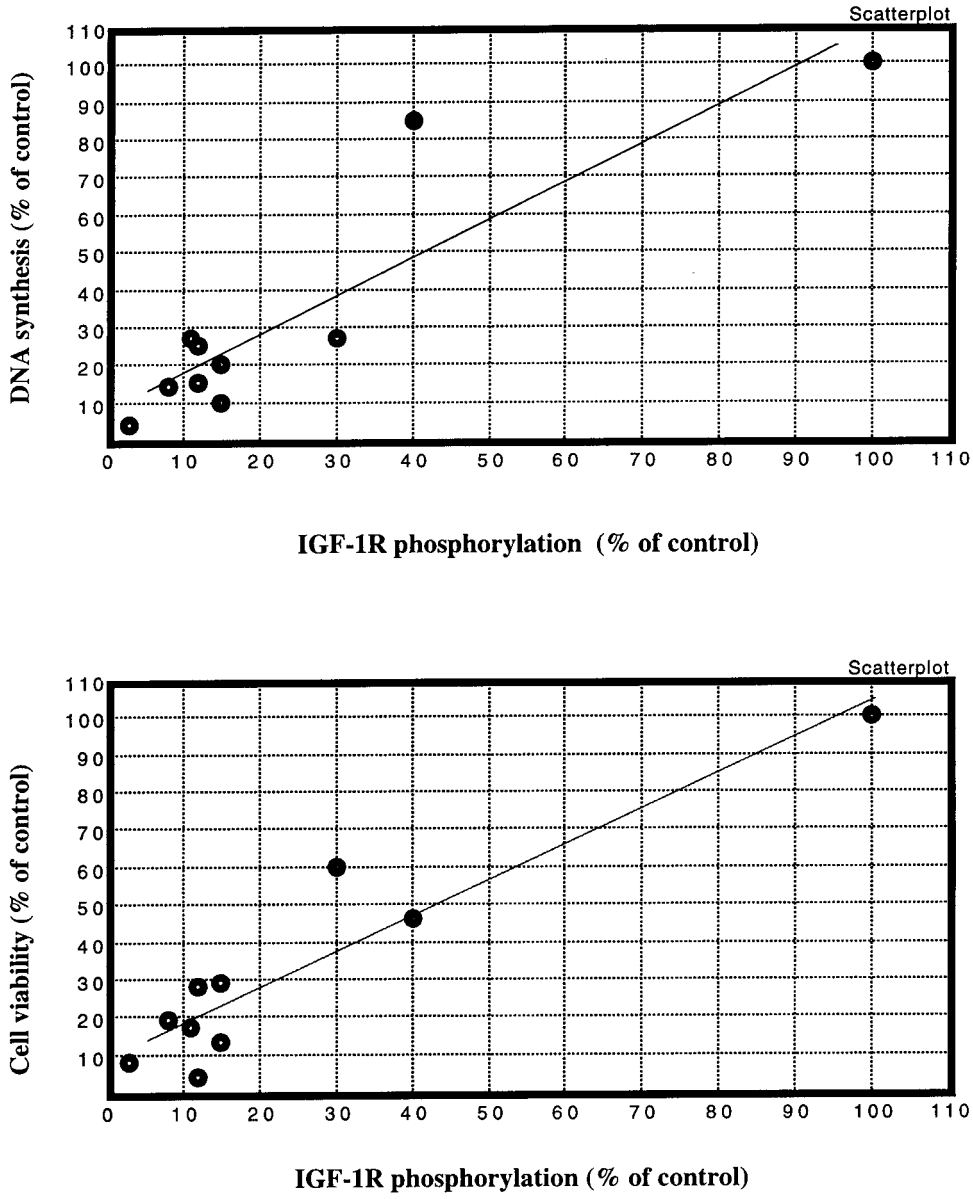


FIGURE 5. Association between IGF-1R phosphorylation and DNA synthesis and cell viability. The amount of phosphorylated β-subunit was quantified by densitometry (from Western blot analysis data shown in Fig. 3), and is indicated as a percentage of control for each cell line. DNA synthesis and cell viability data are obtained from Figures 4B and 4C and are indicated as percentage of control for each cell line. Probability and correlation coefficient for the correlation between IGF-1R phosphorylation and DNA synthesis were less than 0.05 and 0.90, respectively; and for the correlation between IGF-1R phosphorylation and cell viability were less than 0.05 and 0.93, respectively.

quently, this induced growth arrest and cell death in the three investigated uveal melanoma cell lines. In this way, treatment with TM and lovastatin simulate the effect of growth factor depletion, which in itself induces apoptosis in tumor cells.[37] It could be argued that lovastatin and TM kill the cells by other mechanisms than by downregulating the IGF-1R. However, as shown in this study and in others,[10,11,14] the kinetics of the growth inhibition and cell death correlate well with the effects obtained by the decrease in IGF-1R tyrosine phosphorylation, using the IGF-1R blocking monoclonal antibody αIR-3. Furthermore, it has been shown that addition of high concentrations of IGF-1 to cells treated with glycosylation inhibitors can temporarily prevent growth inhibition and apoptosis and that this effect can be abrogated by αIR-3.[10] Similar results have been obtained in other cell lines.[38] These data suggest that downregulation of IGF-1R at the cell surface is an important mechanism by which N-linked glycosylation inhibitors decrease cell growth and survival. Taken together, our results raise the possibility of targeting IGF-1R as a form of treatment in patients with uveal melanoma.

The in vitro results may also support our immunohistochemical data, because we demonstrated a significant association between IGF-1R activity and cell proliferation and cell survival of uveal melanoma cells (see Fig. 5). Deregulated cell growth and suppression of apoptosis represent fundamental properties for propelling the tumor cell and its progeny into

uncontrolled expansion and invasion.[39] Therefore, our experimental data may reflect an association between the level of IGF-1R and the malignant phenotype of uveal melanoma.

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