ABSTRACT

The role of the insulin-like growth factor (IGF) receptor in regulating the growth of melanoma cells was evaluated by examining the effect of antibody-mediated IGF receptor inhibition on the growth of four human melanoma cell lines in culture and in xenotransplants in athymic mice. All four cell lines expressed typical type I IGF receptors and an antibody to this receptor (aIR-3) inhibited [125I]IGF-I binding. However, the cell lines varied widely in their in vitro responsiveness to IGF-I and aIR-3: in the WM 373 and WM 852 cell lines, IGF-I stimulated cell replication and aIR-3 inhibited this response, whereas in the WM 239-A and WM 266-4 cell lines neither the growth factor nor the antibody affected growth. A wide variation was also observed in the effect of the antibody on the growth of the different cell lines as xenotransplants but this qualitatively correlated with the responses observed in vitro: aIR-3 treatment significantly inhibited the growth of the WM 373 and WM 852 xenotransplants but did not inhibit the growth of the WM 239-A or WM 266-4 xenotransplants and may even have had a slight stimulatory effect. These results indicate that the IGF receptor pathway is a functional regulator of the in vivo growth of some melanomas and that this is reflected in the activity of this pathway as determined in vitro. These findings suggest that therapies aimed at inhibiting the IGF pathway may be beneficial in treating some melanomas.

INTRODUCTION

IGF-I and IGF-II are polypeptide hormones which play a fundamental role in normal growth and differentiation (reviewed in Refs. 1 and 2). Functionally, the two IGFs have similar spectra of biological activities and mediate their effects through the same cell surface receptor, the type I IGF receptor (3, 4). The IGFs are synthesized by a variety of cell types and endocrine, paracrine, and autocrine actions have been described. In addition to their well recognized mitogenic activity, the IGFs also stimulate a wide variety of differentiated functions, such as proteoglycan (5), myelin (6), and steroid hormone (7) synthesis in appropriate cell types. In stimulating both differentiated functions and mitogenesis, the IGFs often work synergistically with other trophic hormones and growth factors (7, 8).

In view of their widespread anabolic effects on normal tissues, it is not surprising that a role for the IGFs in tumor growth has been postulated (9–11). The IGFs could stimulate the growth of transformed cells either as a result of their normal mitogenic activity for a particular cell type or because of a specific alteration in the IGF receptor pathway in the transformed cell. In support of a role for the IGFs in the pathophysiology of tumor growth, it has been shown that a variety of tumor types express IGF receptors in vivo (12) and that tumors aberrantly produce IGFs (14, 15). Moreover, type 1 IGF receptor inhibition has been shown to inhibit the growth of Wilms’ tumors (10) and some breast tumor cell lines in vivo (16). However, the role of these hormones in stimulating the growth of most tumor types remains unclear. Although a number of transformed cell lines respond to the IGFs in vitro, many IGF independent cell lines have also been described (9, 17). Furthermore, it is not known whether the IGF responsiveness observed in vitro reflects an absolute dependence on these hormones or whether other growth factors could fulfill this role in vivo. Thus, the role which the IGFs play in the in vivo growth of specific tumor types remains largely undefined.

The present studies were undertaken to define the role of the IGF pathway in stimulating tumor cell replication in vivo and to determine if the in vivo response correlates with that observed in vitro. To this end, we examined the effects of antibody-mediated type I IGF receptor inhibition on the growth of four human melanoma cell lines in culture and as xenotransplants in athymic mice. This cell type was chosen because a variety of human melanoma cell lines that differ in their in vitro responsiveness to IGF are available and this cell type grows readily as s.c. tumors in athymic animals, thus allowing comparison of the in vivo and in vitro responses. The results indicate that the IGF receptor pathway is a functional regulator of the growth of some melanomas in vivo and that this is reflected in the activity of this pathway as determined in vitro.

MATERIALS AND METHODS

Cell Lines, Hormones, and Antibodies. The human melanoma cell lines used in these studies (WM 852, WM 373, WM 266-4, and WM 239-A) were provided by Dr. U. Rodeck (Wistar Institute, Philadelphia, PA) and have been described elsewhere (18, 19). All were obtained from metastatic lesions. The cells were cultured in RPMI 1640 containing 2% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2. Powdered cell culture media (MCDB 104 and RPMI 1640) were purchased from Gibco BRL (Grand Island, NY) and prepared as described previously (3). Human IGF-I was purified from Cohn fraction IV-I as previously reported (3). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

aIR-3, a monoclonal antibody to the human type I IGF receptor (20), was a generous gift of Dr. Steven Jacobs (Wellcome Research Laboratories, Research Triangle Park, NC). This antibody is specific for the human type I IGF receptor and does not bind to the murine type 1 IGF receptor or the human insulin receptor; it is a competitive inhibitor of both IGF-I binding and IGF stimulated DNA synthesis in a variety of normal and transformed human cell types (3, 10, 16, 21). The aIR-3 used in these studies was produced as ascites fluid and purified on protein-A Sepharose (3). A mouse monoclonal antibody to the major surface glycoprotein of human Pneumocystis carinii, aPC, which is of the same subclass as aIR-3 (IgG2a), was used as a control in some of these studies. This antibody was a gift of Dr. Francis Gigliotti (University of Rochester, Rochester, NY) and was purified as described for aIR-3.

In Vitro Cell Replication Studies. To assess the role of the IGF-receptor pathway in stimulating the growth of human melanoma cell lines in vitro, a modification of the serum-free system previously described for WI-38 fibroblasts (3) was used. Cells from stock cultures were plated into 16-mm (24-well) cluster dishes at a density of 20,000–80,000 cells/well in 1.0 ml RPMI 1640 supplemented with glutamine and 2% fetal bovine serum. After 24 h, the medium was removed and the cells were washed twice with PBS and 1.0 ml serum-free MCDB 104 medium was added. After 48 h, the medium was removed and 1.0 ml fresh MCDB 104 containing 0.1% bovine serum albumin, penicillin (100 units/ml), streptomycin (100 µg/ml), transferrin (1 µg/ml),
dexamethasone (0.10 μM), and the desired concentration of growth factor, antibody, or serum was added to replicate wells. This medium was removed and replaced with fresh medium and reagents every 2 to 3 days. After 3–7 days, triplicate wells were harvested by trypsinization and the cell numbers were determined by electronic counting (Coulter Electronics, Hialeah, FL). All in vitro experiments were repeated at least three times with similar results.

Chemical Cross-linking of \(^{125}\text{I}\)IGF-I to Monolayer Cells. IGF-I was iodinated to a specific activity of 140 μCi/μg using chloramine-T (22). \(^{125}\text{I}\)IGF-I was chemically cross-linked to monolayer cultures of cells using 0.1 mM disuccinimidyl suberate as previously described (9). After cross-linking, the monolayers were solubilized with buffer containing 0.1% SDS and resolved by SDS-polyacrylamide gel electrophoresis using the discontinuous buffer system of Laemmli (23). The stained dried gels were autoradiographed and band intensity was quantitated by laser densitometry.

In Vivo Experiments. These studies were approved by the Committee on Animal Resources, University of Rochester. Adult female athymic mice (BALB/c nu/nu, Taconic Farms, Germantown, NY) weighing 20–30 g were inoculated s.c. in the flank with 1–2.1 × 10^7 log phase cells. At the same time an i.p. injection of 500 μg αIR-3, αPC, or an equal volume of PBS was administered and continued twice weekly for the remainder of the experiment. For those cell lines which grew as spherical tumors, length (l) and width (w) were measured once or twice weekly using calipers and the mean diameter (d) was calculated using the formula:

\[ d = \frac{1}{2} (l \times w)^{1/2} \]

At the conclusion of the experiment the mice were killed by cervical dislocation, the tumors were removed by dissection, and their wet weights were recorded. Representative portions of tumor were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin. Multiple areas were examined with a Spectra Services Biological Analysis System and the total number of cells/unit area was determined. In vivo experiments were repeated twice for each cell line.

Statistical Analysis. Differences between groups were assessed by non-paired t testing.

RESULTS

Binding of \(^{125}\text{I}\)IGF-I to Human Melanoma Cell Lines. Chemical cross-linking of \(^{125}\text{I}\)IGF-I using the bifunctional cross-linking reagent disuccinimidyl suberate indicated that each of the human melanoma cell lines examined in this study expresses type I IGF receptors with characteristics similar to those reported for other nonneuronal cell lines (data not shown) (9, 24, 25). Specifically, when the cross-linked material was subject to SDS-polyacrylamide gel electrophoresis under reducing conditions, a radiolabeled band with an apparent approximate M₀, 130,000 was observed; this size is characteristic of the α subunit of the type I receptor on nonneuronal cells. The intensity of this band was decreased by 92–94% when 4.6 nM unlabeled IGF-I was included, indicating that the band is not the result of \(^{125}\text{I}\)IGF-I binding to the closely related insulin receptor. Inclusion of 100 nM αIR-3 decreased the intensity of the M₀, 130,000 band 64–86% in the different cell lines. With the WM 239A, WM 266–4, and WM 373 cell lines radiolabeled bands in the M₀, 26,000 to 40,000 range were also observed; binding to these bands was abolished by unlabeled IGF-I but was unaffected by αIR-3 or insulin, indicating that they were due to binding of \(^{125}\text{I}\)IGF-I to IGF binding proteins produced by the cells.

In Vitro Cell Replication Studies. Under the in vitro conditions used in these experiments, three different growth patterns were observed. The first pattern was observed with the WM 373 cell line; this cell line did not proliferate in serum-free medium lacking IGF-I but cell numbers increased up to 8-fold when the medium was supplemented with nanomolar concentrations of IGF-I or micromolar amounts of insulin (Fig. 1). The mitogenic effects of both IGF-I and insulin were competitively inhibited by αIR-3, indicating that both hormones operate through the type I IGF receptor in these cells. The second growth pattern was observed with the WM 852 cell line; these cells continued to proliferate in serum-free medium lacking exogenously added growth factors but their rate of growth was increased approximately 2-fold by nanomolar concentrations of IGF-I or micromolar concentrations of insulin (Fig. 2). Although the basal rate of proliferation of these cells was not affected by αIR-3, the mitogenic effects of both IGF-I and insulin were inhibited by the antibody, implicating the type I IGF receptor in mediating these responses. The third pattern was observed with the WM 239A (Fig. 3) and WM 266–4 (data not shown) cell lines; these cells continued to proliferate in serum-free medium lacking exogenously added growth factors and neither IGF-I nor insulin further stimulated cell replication. In both of these lines, αIR-3 had no effect on proliferation when present either alone or with IGF-I or insulin.

In Vivo Experiments. To assess the role of the IGF pathway in stimulating the growth of human melanomas in vivo, athymic mice were inoculated s.c. with tumor cells and the effect of αIR-3 on tumor growth was monitored. With two cell lines, WM 373 (Fig. 4) and WM 852 (Fig. 5), αIR-3 treatment significantly inhibited tumor growth.
When inoculated s.c. the WM 373 cells grew as flat, darkly pigmented tumors. Because it was impossible to accurately estimate the thickness of these tumors, their volumes could not be calculated and analysis was limited to comparisons of tumor wet weights measured at the time of sacrifice. The results of two experiments are shown in Fig. 4; in both, the tumor weights were significantly less in the aIR-3 treated than in the control groups ($P < 0.01$). When inoculated s.c., the WM 852 cells grew as approximately spherical tumors, allowing for mean diameter estimations. Treatment of animals bearing the WM 852 melanoma with aIR-3 inhibited tumor growth and prolonged treatment resulted in the disappearance of palpable (but not microscopic) tumors (Fig. 5). As expected from its species specificity, aIR-3 had no effect on the tumor-free body weights of the treated animals (data not shown).

In contrast to its effects on the WM 373 and WM 852 xenotransplants, aIR-3 did not inhibit the growth of either the WM 266-4 (Fig. 6) or WM 239-A xenotransplants (Fig. 7). The latter tumors also grew more rapidly and reached larger diameters. In mice bearing the WM 266-4 melanoma, tumor diameter increased approximately 2-fold between days 3 and 20 with no difference between the aIR-3 treated and the control animals. In both experiments with the WM 239-A cell line, mean tumor diameters were actually larger in the animals receiving aIR-3 than in the control animals but these differences did not reach statistical significance. Again, aIR-3 had no effect on the tumor-free body weights in any of these experiments (data not shown).

Histological features of the xenotransplants are summarized in Table 1. The cell morphologies of the xenotransplants did not correspond to those reported for the original tumors (18, 19). In particular, the WM 373 xenotransplants were epitheloid whereas the original tumor was reported to have spindle cell morphology (18) and the WM 852 xenotransplants had spindle cell morphology while the original tumor was epitheloid (19). aIR-3 treatment had no effect on either cell morphology or pigment production by the tumors. Lymphocytic infiltration was not present in any of the tumors from animals which received either the control antibody or PBS or in the tumors which responded to aIR-3 treatment (i.e., WM 373 and WM 852). Somewhat surprisingly, however, the nonresponding xenotransplants exposed to aIR-3 showed slight (WM 239-A) to moderate (WM 266-4) lymphocytic infiltration. Histological examination also suggested a decreased cellularity, prevalence of mitotic figures, and invasiveness in the WM 373 and WM 852 tumors from animals which received aIR-3. In contrast, there were no obvious differences in these parameters between the aIR-3 treated and control groups for the WM 266-4 and WM 239-A tumors. When the tumors were examined using quantitative morphometric analysis (Table 1) the WM 373 and WM 852 tumors from the animals receiving aIR-3 had lower cell concentrations (number of cells/mm$^2$ of tumor) than the tumors from the animals in the control groups ($P < 0.05$). In contrast, tumors derived from the WM 266-4 and WM 239-A lines showed an increased cell concentration when exposed to aIR-3 compared to the controls ($P < 0.05$).

DISCUSSION

The present studies were undertaken to define the role of the IGF pathway in stimulating tumor cell replication in vivo and to determine if the in vivo response correlated with that observed in vitro. To this end, we examined the effect of antibody mediated type I IGF receptor inhibition on the growth of four human melanoma cell lines in culture and as xenotransplants in athymic mice. The results indicate that the IGF receptor pathway is an important regulator of the growth of some melanoma cell lines in vivo and that this is reflected in the activity of this pathway as determined in vitro.

The human melanoma cell lines examined in this study all express type I IGF receptors with physical characteristics and binding specificities similar to those reported for other nonneuronal cell types. In all cell lines, aIR-3, a monoclonal antibody specific for the human type I IGF receptor, inhibited IGF-I binding. However, the cell lines varied widely in their in vivo responsiveness to IGF-I and aIR-3. The growth of the WM 373 cell line was IGF-I dependent: these cells became quiescent in medium lacking IGF-I and proliferated in response to nanomolar concentrations of the growth factor. Moreover, aIR-3 competitively inhibited the mitogenic effect of IGF-I on these cells. In contrast, the WM 852 cell line was responsive to IGF-I but its growth
αIR-3 on the growth of the melanomas as xenotransplants. A wide variation was observed in the effects of the antibody on the growth of the different xenotransplants but this qualitatively correlated with the responses observed in vitro. For the WM 373 and WM 852 xenotransplants, αIR-3 treatment inhibited tumor growth significantly. Because of their morphology, the volumes of the tumors derived from the WM 373 cell line were difficult to estimate and tumor wet weight at sacrifice had to be used for comparisons; this measurement probably overestimated tumor size because the tumor tissue could not be dissected completely free from adjacent connective tissue elements. Macroscopically, tumor identification was only possible because the cells were darkly pigmented. Nevertheless, significant inhibition of tumor growth was observed in the animals receiving αIR-3. Similarly, αIR-3 treatment inhibited the growth of the WM 852 xenotransplants and prolonged therapy resulted in tumor regression, although histological examination indicated that tumor cells were still present. The marked regression which occurred in the animals which received αIR-3 was somewhat surprising since the antibody did not inhibit the basal growth of these cells in vitro. This suggests that additional regulatory pathways are functioning to inhibit the growth of these cells in vivo. By contrast, in the WM 239-A and WM 266-4 xenotransplants, αIR-3 treatment did not inhibit tumor growth and may even have had a slight stimulatory effect. Thus, the IGF-receptor pathway is a functional regulator of the in vivo growth of some, but not all, melanoma cell lines and this is reflected in the activity of this pathway as determined in vitro.

Two previous studies have investigated the effects of αIR-3 on the growth of tumor xenotransplants (10, 16). In one study, αIR-3 was not dependent on it; these cells continued to proliferate in defined medium lacking IGF-I but their rate of proliferation increased in response to the growth factor. αIR-3 did not affect the basal rate of proliferation of WM 852 cells, indicating that basal growth was not the result of autocrine IGF production; however, αIR-3 did inhibit the effect of exogenous IGF-I on WM 852 cells. Finally, the in vitro growth of the WM 266-4 and WM 239-A cell lines was independent of IGF-I; these cell lines continued to proliferate in serum-free medium lacking IGF-I and addition of the growth factor or αIR-3, alone or in combination, had no significant effect on growth. As we did not determine if these cell lines produce IGF in vitro, it is possible that this independence is the result of autocrine IGF action. However, we believe this is unlikely since the concentration of αIR-3 used in these studies (100 nm) is 20-fold greater than that which inhibits the growth of other cell lines known to be dependent on autocrine IGF production (26). At any rate, no correlation was observed between the presence of type I IGF receptors on melanoma cell lines and their mitogenic response to the hormone in vitro. However, a correlation was observed between the ability of IGF-I (or insulin) to stimulate cell replication and the ability of αIR-3 to inhibit this response.

To investigate the role of the IGF pathway in stimulating tumor growth in vivo, we examined the effect of passive immunization with αIR-3 on the growth of the melanomas as xenotransplants. A wide variation was observed in the effects of the antibody on the growth of the different xenotransplants but this qualitatively correlated with the responses observed in vitro. For the WM 373 and WM 852 xenotransplants, αIR-3 treatment inhibited tumor growth significantly. Because of their morphology, the volumes of the tumors derived from the WM 373 cell line were difficult to estimate and tumor wet weight at sacrifice had to be used for comparisons; this measurement probably overestimated tumor size because the tumor tissue could not be dissected completely free from adjacent connective tissue elements. Macroscopically, tumor identification was only possible because the cells were darkly pigmented. Nevertheless, significant inhibition of tumor growth was observed in the animals receiving αIR-3. Similarly, αIR-3 treatment inhibited the growth of the WM 852 xenotransplants and prolonged therapy resulted in tumor regression, although histological examination indicated that tumor cells were still present. The marked regression which occurred in the animals which received αIR-3 was somewhat surprising since the antibody did not inhibit the basal growth of these cells in vitro. This suggests that additional regulatory pathways are functioning to inhibit the growth of these cells in vivo. By contrast, in the WM 239-A and WM 266-4 xenotransplants, αIR-3 treatment did not inhibit tumor growth and may even have had a slight stimulatory effect. Thus, the IGF-receptor pathway is a functional regulator of the in vivo growth of some, but not all, melanoma cell lines and this is reflected in the activity of this pathway as determined in vitro.

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Two previous studies have investigated the effects of αIR-3 on the growth of tumor xenotransplants (10, 16). In one study, αIR-3 was
shown to inhibit the growth of three different human Wilms' tumor xenotransplants (10); it also inhibited the growth of each of these cell lines in vitro, supporting a correlation between in vitro and in vivo responsiveness. Another study examined the effects of aIR-3 on the growth of breast tumor xenotransplants in vivo and in vitro (16). In this study aIR-3 was found to inhibit both the in vivo and in vitro growth of the estrogen independent MDA 231 cell line but to inhibit only the in vitro growth of the estrogen responsive MCF-7 cell line. However, the in vivo growth of the MCF-7 cell lines was dependent upon estrogen, whereas the in vitro studies did not examine the effect of estrogen on aIR-3 responsiveness. Estrogen stimulates autocrine IGF production by MCF-7 cells in vitro (27); it is therefore possible that the different responses observed in vivo and in vitro were due to estrogen stimulation of autocrine IGF production to levels sufficient to overcome the inhibitory effect of aIR-3. Alternatively, estrogen could activate an IGF independent growth stimulatory pathway in MCF-7.

There are a number of mechanisms by which aIR-3 could inhibit the growth of the xenotransplants. These include: (a) direct inhibition of type I IGF receptor activity as occurs in vitro; (b) indirect inhibition resulting from activation of immunological mechanisms, such as macrophage or complement mediated cytotoxicity; and (c) nonspecific inhibition resulting from the presence of a toxin in the antibody preparation. We believe indirect mechanisms are unlikely because such reactions are not usually observed with the IgG antibody subclass, no evidence of an inflammatory response was observed in the aIR-3 responsive tumors, and such inhibition would be expected to affect all four cell lines as they all bound the antibody. A nonspecific mechanism is also unlikely since oPC, a control antibody which was purified sequentially with aIR-3, did not inhibit the growth of the three xenotransplants where it was used as a control. Therefore, we feel that the most likely mechanism by which aIR-3 inhibits the growth of WM 373 and WM 852 xenotransplants is by directly inhibiting type I IGF receptor activity.

While the observations presented here suggest that IGF receptor inhibition may be beneficial in the treatment of some melanomas, the results must be interpreted with caution. First, the proportion of spontaneously occurring tumors which might be IGF responsive is unknown. The cell lines examined in this report were selected, in part, because of differences in their in vitro responsiveness to IGF-I and they may not be representative of a large population of melanomas. Second, the melanomas examined in this study were first cultivated in vitro. This process may select for cells which have an altered growth factor responsiveness. Third, the observation that aIR-3 appeared to have a modest stimulatory effect on the in vivo growth of two of the cell lines suggests that such therapy could also have deleterious effects. Finally, the problems inherent in administering large quantities of a monoclonal antibody which can react with receptors present on normal (human) cells are likely to be significant. Nevertheless, the results observed in this study suggest that therapies aimed at modulating IGF receptor function or the local bioavailability of the IGFs may be beneficial in selected patients with melanoma.

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Effects of Insulin-like Growth Factor Receptor Inhibition on Human Melanomas in Culture and in Athymic Mice

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