Dependence of a Human Squamous Carcinoma and Associated Paraneoplastic Syndromes on the Epidermal Growth Factor Receptor Pathway in Nude Mice

Toshiyuki Yoneda, Maria M. Alsina, Kazuya Watatani, Francoise Bellot, Joseph Schlessinger, and Gregory R. Mundy

Division of Endocrinology and Metabolism, Department of Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284; T. Y., M. M. A., K. W., G. R. M.; Rorer Biotechnology Inc., King of Prussia, Pennsylvania 19406; F. B.; and Department of Pharmacology, New York University, New York, New York 10016. [J. S.]

ABSTRACT

Increased levels of epidermal growth factor receptor (EGFR) have been shown on squamous cell carcinomas. Recently, we described a squamous cell carcinoma (MH-85) derived from the oral cavity which was associated with several paraneoplastic syndromes including hypercalcemia and cachexia. This tumor induced the same paraneoplastic syndromes in nude mice (BALB/c, nu/nu, male, 4-6 weeks old). Scatchard analysis revealed that there are two classes of EGFR in MH-85. The dissociation constant and number of binding sites for the high affinity receptors were 38 pm and 5 x 10^5/cell, respectively, and 2.2 nm and 6 x 10^6 cell, respectively, for the low affinity receptors. Growth of MH-85 in culture was stimulated by epidermal growth factor (EGF) and inhibited by monoclonal antibody 108 to human EGFR, which recognizes the extracellular domain of the EGFR receptor. Surgical removal of submandibular glands from male nude mice resulted in a dramatic decrease in plasma EGF levels and a significant reduction of tumor growth, hypercalcemia, and cachexia. When EGF (5 ug/mouse, every 2 days for 6 weeks, i.p.) was administered to these sialoadenectomized animals, tumor growth increased, with a parallel increase in hypercalcemia. When monoclonal antibody 108 (1 mg/mouse, i.p.) was given 1, 5, and 10 days after MH-85 tumor implantation, tumor formation was retarded, which resulted in delayed onset of hypercalcemia and cachexia. Moreover, when the antibody was injected 6 times in nude mice exhibiting large tumors and profound hypercalcemia and cachexia, there was a striking decrease in tumor growth, which was accompanied with a reversal of hypercalcemia and cachexia. These results indicate that growth of the human squamous cell carcinoma MH-85 is dependent on the EGFR pathway and that subsequent development of hypercalcemia and cachexia is dependent on tumor growth. They also suggest that agents which interfere with the EGFR pathway may have therapeutic potential as anticancer agents in some human tumors.

INTRODUCTION

It is well established that the EGFR signal transduction pathway is important in the growth of many human tumors (1, 2). Overexpression of EGFR is common in a variety of human epithelial malignancies (3), including breast (4-6), esophagus (7), bladder (8), lung (9), female genital tract (10), brain (11, 12), and head and neck (13-16). Expression of EGFR in these tumors is related to cell growth and tumorigenicity (17), and blocking of this pathway with monoclonal antibodies against EGFR inhibits tumor growth (18-24). TGF-α, which is also a ligand for the EGFR, is frequently produced by malignant as well as normal cells and may stimulate their own proliferation (25-27); thus, it has been implicated as an autocrine factor in tumor growth in vivo (28, 29). However, less is known about the role of EGF itself in oncogenesis. EGF is rarely if ever produced by human tumors and, therefore, is unlikely to be an autocrine factor (29). In this report, we have identified a human squamous cell carcinoma, derived from the maxillary sinus, which was dependent on the EGFR signal transduction pathway for its growth both in vitro and in vivo. The cell line (MH-85) established from this tumor overexpressed EGF. EGF stimulated and mAb108 inhibited MH-85 growth in culture. In nude mice which underwent surgical removal of the submandibular glands, a major source of EGF in male mice, tumor growth was diminished, which was reversed by injection of EGF. Furthermore, mAb108 inhibited MH-85 tumor growth in nude mice. We conclude that growth and associated paraneoplastic syndromes of this human squamous cell carcinoma in nude mice are under the control of EGF and the EGFR pathway.

MATERIALS AND METHODS

MH-85 Tumor and Cells. The MH-85 tumor was isolated from a patient with a squamous cell carcinoma in the maxillary sinus, who manifested marked leukocytosis (180,000/mm^3, >90% mature granulocytes), cachexia (loss of body weight, muscle, and fat), and hypercalcemia (13.0 mg/dl) (30). Nude mice transplanted with this tumor also develop profound hypercalcemia, cachexia, and leukocytosis 6 weeks after transplantation (31).

MH-85 cells were grown in aMEM (Hazleton Biologies, Inc., Lenexa, KA) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Inc., Logan, UT) and 1% penicillin-streptomycin solution (GIBCO, Grand Island, NY).

125I-EGF Binding Assay. Confluent MH-85 cells in 24-well plates were placed on ice, washed twice with ice-cold binding medium (aMEM supplemented with 2 mg/ml bovine serum albumin and 25 mM 4(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.4), and incubated in the binding medium for more than 2 h at 4°C. The cells were then incubated with different concentrations of 125I-EGF (76.6 μCl/μg; ICN Radiochemicals, Irvine, CA), in the presence or absence of 200-fold excess unlabeled EGF, for 2 h at 4°C. At the end of the incubation, the cells were washed 5 times with ice-cold binding medium and solubilized with lysis buffer (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% sodium dodecyl sulfate), and radioactivity was counted.

Colonoy Assay for MH-85 Growth. MH-85 cells (100/17-mm well) were seeded in plastic tissue culture plates (24-well plates; Corning Glassware, Corning, NY) and cultured overnight in aMEM supplemented with 10% FCS. The cells were then cultured in aMEM supplemented with 0.2% FCS, in the presence or absence of varying concentrations of EGF (Collaborative Research, Bedford, MA) and mAb108, for 8-10 days. At the end of the culture, the plates were washed with Ca/
Mg-free PBS, fixed with 4% (v/v) formaldehyde in Ca/Mg-free PBS for 15 min, and stained with hematoxylin as described (22). The number of colonies containing more than 20 cells was counted under the microscope.

MH-85 Tumor Implantation into Nude Mice. MH-85 tumor (3 mm in diameter) was implanted s.c. in the right dorsal area of 4- to 6-week-old male BALB/c nu/nu mice (Harlan Sprague-Dawley, Indianapolis, IN). Animals were fed, ad libitum, an autoclaved diet (Teklad-LDH-485; calcium, 0.83 mg/kg; phosphorus, 0.65 mg/kg; Harlan Sprague-Dawley) and tap water (acidified to pH 2.5). The body weights of MH-85 tumor-bearing animals and their tumor sizes were measured using anesthesiad with ether. Tumor size was calculated as 4/3 π(long axis + short axis−1)/23, as described previously (32).

Determination of Blood Ionized Calcium Levels. Thirty μl of blood were drawn from the orbital plexus with calcium-titrated sodium heparinate-coated tubes (Corning, Halstead, Essex, England), using anesthesia with ether. Blood ionized calcium levels were determined using a Chiba-Corning calcium-pH analyzer (model 634; Corning, Medfield, MA).

Sialoadenectomy. Bilateral submandibular glands of 4- to 6-week-old male nude mice were aseptically removed after vertical midline incisions, approximately 1 cm in length, were made on the skin of the neck region, using anesthesia with Nembutal (0.05 mg/g body weight). Control animals received the same cutaneous incision. Cutaneous incisions were closed by Autoclips (Clay Adams, Parsippany, NY).

Determination of Plasma EGF Levels. Blood was drawn by cardiac puncture and centrifuged to isolate plasma. EGF in the plasma was extracted according to the methods described (33), with slight modifications. Plasma was acidified with acetic acid at a final concentration of 3%, diluted with 0.1% trifluoroacetic acid by 5-fold, and applied to disposal Sep-Pack C18 columns (Waters Associates, Milford, MA), which were equilibrated with 10% acetonitrile and 0.1% trifluoroacetic acid. EGF was eluted with 40% acetonitrile containing 0.1% trifluoroacetic acid. The elutes were concentrated to dryness in a Speed-Vac (model 2439). The number of colonies containing more than 20 cells was counted under the microscope.

RESULTS

125I-EGF Binding to MH-85 Cells. MH-85 demonstrated dose-dependent and saturable binding of EGF (Fig. 1a). Scatchard analysis of the binding data indicated the presence of two populations of receptor, with different affinities for EGF. The receptor with lower affinity has a dissociation constant of 38 pM and an estimated number of binding sites of 5 x 10^4/cell. These values are comparable to those of other human epidermal cancer cell lines such as A431 (35) and KB (36) cells, which are known to possess overexpressed EGFR with high affinity.

Effect of EGF and mAb108 on MH-85 Growth in Culture. EGF as low as 10 ng/ml significantly increased colony formation of MH-85 (Fig. 2). On the other hand, mAb108 decreased colony formation of MH-85 in a dose-dependent manner, both in the presence and in the absence of EGF (50 ng/ml) (Fig. 3a).

Equal concentrations of control mAb7H01 showed no effect (Fig. 3b).

Effects of Sialoadenectomy on MH-85 in Nude Mice. The results described above indicated that MH-85 growth in culture is modulated by EGF. To study this, submandibular glands of male nude mice were removed 7 days prior to MH-85 implantation. It is known that more than 95% of EGF is produced in submandibular glands in male mice (37). Consistent with these previous reports, plasma EGF levels of sialoadenectomized male nude mice at the time of MH-85 tumor implantation (7 days after surgery) dramatically decreased (0.09 ± 0.08 ng/ml), compared to those of sham-operated animals (2.01 ± 1.4 ng/ml) (Fig. 4). Plasma EGF levels of sialoadenectomized animals with established MH-85 tumors at 10 weeks after tumor implantation increased but were still much lower than those of sham-operated animals (sham-operated = 2.8 ± 1.2, sialoadenectomized = 0.23 ± 0.14 ng/ml, n = 6 and 7, respectively). The data also demonstrate that carrying MH-85 tumors did not significantly change plasma EGF levels in sham-operated (week 0 = 2.01 ± 1.4, week 10 = 2.8 ± 1.2 ng/ml, n = 13 and 6, respectively) and sialoadenectomized (week 0 = 0.09 ± 0.08, week 10 = 0.23 ± 0.14 ng/ml, n = 8 and 7, respectively) nude mice. Non-tumor-bearing nude mice with no treatment did not show significant changes in plasma EGF levels at week 0 (2.4 ± 1.3 ng/ml, n = 4) and week 10 (2.1 ± 0.9 ng/ml, n = 4).
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Colony No/well (Mean ± S.E., n = 4)

![Bar chart showing colony numbers](chart.png)

**EGFR MoAb (μg/ml)**

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**Fig. 3. a, effect of mAb108 on colony formation by MH-85 cells in the presence (○) or absence (□) of EOF (50 ng/ml). b, effect of mAb108 (□) and mAb7H01 (control ascites) (○) on colony formation of EOF (50 ng/ml)-stimulated MH-85 cells. Experiment was performed in the same way as described in the legend to Fig. 2. * significantly different than cultures without antibodies (P < 0.01).**

**4-1 3 2 1**

**EOF (ng/ml)**

**Sham Sialoadenectomy**

**Fig. 4. Plasma EGF levels in sham-operated (○) and sialoadenectomized (□) nude mice at the time of MH-85 tumor implantation. Plasma EGF was determined as described in “Materials and Methods.” Values are mean ± SE. Numbers in parentheses indicate the number of animals examined. Sham-operated animals demonstrated significantly higher levels of plasma EGF (P < 0.05).**

**As shown in Fig. 5a, MH-85 grew to approximately 20 cm³ in size in sham-operated animals 10 weeks after tumor implantation. When MH-85 was implanted into sialoadenectomized male nude mice with decreased plasma EGF levels, there was a marked decrease in tumor growth (P < 0.01). However, injection of 5 μg of EGF every 2 days for 6 weeks into sialoadenectomized animals completely restored MH-85 growth. Plasma EGF levels in these animals at the time of sacrifice (5 days after last injection of EGF) were 3.6 ± 1.8 ng/ml (n = 5).**

**Sham-operated animals bearing MH-85 tumor showed a profound decrease in body weight due to cachexia, compared to non-tumor-bearing animals (P < 0.01) (Fig. 5b). However, there was less decrease in body weight in sialoadenectomized nude mice bearing MH-85 tumor than in sham-operated nude mice with MH-85 tumor (P < 0.05). Although EGF restored MH-85 tumor growth in sialoadenectomized nude mice, these animals did not manifest cachexia to the same degree as sham-operated animals.**

**Sham-operated animals bearing MH-85 tumor manifested marked hypercalcemia (Fig. 5c). The hypercalcemia was significantly diminished in sialoadenectomized animals (P < 0.01), due to decreased tumor burden. When EGF was injected into sialoadenectomized animals for 6 weeks, marked hypercalcemia identical to that seen in sham-operated animals developed, resulting from restored tumor growth. The same dose of EGF did not increase blood ionized calcium levels in non-tumor-bearing animals (data not shown).**

**Effects of mAb108 on MH-85 in Nude Mice. In the first set of experiments, nude mice were given injections of mAb108 or mAb7H01 (1 mg/mouse, i.p.) at 1, 5, and 10 days after tumor inoculation. With mAb108, tumor growth was markedly delayed (Figs. 6, center, and 7a). Tumor were not palpable until 60 days after tumor inoculation, compared with 20 days for animals given injections of mAb7H01. Because of this delay of MH-85 tumor development, the onset of hypercalcemia was retarded (Fig. 7b) and no cachexia occurred (Figs. 6, center, and 7c). Dependency of these paraneoplastic syndromes on MH-85 growth was previously demonstrated (31).**

**In the second set of experiments, cachectic and hypercalcemic nude mice bear large MH-85 tumors were given injections of mAb108 or mAb7H01 60 days after tumor inoculation. With mAb108, continued tumor growth was halted, and there was a slight regression in tumor growth (Figs. 6, right, and 8a). As a result of the changes in tumor growth, blood ionized calcium levels started to decrease (Fig. 8b) and the body weight of the animals started to increase (Figs. 6, right, and 8c).**

**In both sets of experiments, nude mice given injections of mAb108 showed more activity in their behavior and food intake than did nude mice given injections of mAb7H01 (data not shown), suggesting that mAb108 at this dose (1 mg/mouse) is not harmful to tumor-bearing nude mice.**

**Fig. 5. Effects of sialoadenectomy on MH-85 tumor size (a), body weight of MH-85 tumor-bearing nude mice (b), and calcium levels (c). ○, sham-operated; □, sialoadenectomized; ■, sialoadenectomized plus EGF (5 μg/mouse, every 2 days for 6 weeks, i.p.); ▲, non-tumor-bearing. Values are mean ± SE of two separate experiments (n = 9 for each treatment group).**
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Fig. 6. Photograph of MH-85 tumor-bearing animals injected with mAb7H01 (control ascites) (left), mAb108 at 1 mg/mouse on days 1, 5, and 10, i.p. (center), or mAb108 1 mg/mouse, every 2 days 5 times from day 40, i.p. (right). The picture was the most representative and was taken 60 days after tumor implantation. Note (a) large tumor formation, decrease in body weight (21 g), and loss of muscle and adipose tissue in the animal treated with mAb7H01 (control ascites) (left); (b) marked decrease in tumor growth, no decrease in body weight (31 g), and no loss of muscle and adipose tissue in the animal treated with mAb108 on days 1, 5, and 10 (center); and (c) significant decrease in tumor size due to necrosis and recovery from decrease in body weight (30 g) and loss of muscle and adipose tissue in the animal treated with mAb108 from day 40 (right). Blood ionized calcium levels in left, center, and right animals were 2.49, 1.21, and 1.43 mmol/L (normal range, 0.95–1.28 mmol/L), respectively.

Tumor Size (mm$^3$ x 10$^3$) Ca$^{2+}$ (mmol/L) Body weight (g) treatment of nude mice with mAb108 not only delays MH-85 tumor formation but also inhibits progressive tumor growth. These results indicate that growth of MH-85 in nude mice is modulated by EGF and the EGFR pathway.

More recently, it has also been shown that tyrphostins, small molecular weight compounds which inhibit EGF-dependent tyrosine kinase, are capable of influencing tumor cell proliferation in vitro (38–41). In a preliminary study, we have found

DISCUSSION

In the present study, we have examined an established cell line, MH-85, derived from a human squamous cell carcinoma, both in vitro and in vivo. We have found that the cells overexpress EGFR and they are responsive to EGF and mAb108. We have shown that in male nude mice removal of the submandibular glands decreases plasma EGF levels, which is accompanied by significant diminution of MH-85 tumor growth. The decreased tumor growth in these sialoadenectomized animals can be reversed by injection of EGF. We have also shown that

Fig. 7. Effects of mAb108 tumor size (a), blood ionized calcium levels (b), and body weight (c). mAb108 (1 mg/mouse) was given on days 1, 5, and 10 after tumor implantation, as indicated by arrows. •, animals given injections of mAb7H01 (control ascites); ○, animals given injections of mAb108. Values shown are mean ± SE of six animals for each treatment group. *, significantly different from corresponding control (P < 0.05).

Fig. 8. Effects of mAb108 on tumor size (a), blood ionized calcium levels (b), and body weight (c). mAb108 (1 mg/mouse) was given every 2 days 4 times and every 3 days twice from day 60, when MH-85-bearing animals manifested large tumor, marked hypercalcemia, and cachexia, as indicated by arrows. •, animals given injections of mAb7H01 (control ascites); ○, animals given injections of mAb108. Twenty-four animals were inoculated with MH-85 tumor at day 0 and divided into two groups (mAb108- and mAb7H01-treated groups) at day 60. Values are mean ± SE of three separate experiments (n = 24 until day 60 and n = 12 for each treatment group thereafter). *, significantly different from corresponding control (P < 0.05).
that one of these compounds (38) has growth-inhibitory effects on MH-85. Together with data which show that transfection of cells with mutated EGFR influences their growth in vivo, depending on the capacity of these receptors to increase tyrosine kinase activity (42), this shows that the EGF signaling pathway is clearly important in MH-85 tumor growth.

Our findings indicate the dependence of MH-85 tumors on EGF or factors which interact with the EGFR. In the present study, mAb108 decreased the growth of unstimulated as well as EGF-stimulated MH-85 cells, suggesting that this antibody interferes with the binding to the EGFR of autocrine growth factor(s) secreted by MH-85. We could not detect significant levels of EGF in MH-85 culture supernatants by using the radioimmunoassay described above (data not shown). This is consistent with previous findings that very few human tumors produce EGF (28, 29). As another likely candidate for an autocrine growth factor, we measured TGF-α levels by using an enzyme-linked immunosorbent assay kit (Triton Biosciences, Alameda, CA). We found 5–35 pg/ml TGF-α in MH-85 culture supernatants. Although we do not know if these levels of TGF-α are sufficient to cause MH-85 tumor growth, TGF-α produced by MH-85 may play a role as an autocrine or paracrine growth factor in this tumor.

Previous reports have shown that anti-EGFR monoclonal antibodies markedly inhibit tumor formation when administration is started on the day of tumor implantation (18, 22). However, in these experiments the antibodies are not able to decrease growth of established tumors, although they inhibit the further growth of the tumors. This is largely due to clonal heterogeneity of established tumors, which are composed of cells dependent or independent on EGF or TGF-α. In our study, mAb108 given only 3 times immediately after tumor inoculation dramatically suppressed tumor formation. Furthermore, six injections of mAb108 to animals with established tumors not only inhibited further enlargement of the MH-85 tumor but also decreased its size. This result suggests that a major population of cells in the MH-85 tumor is dependent on the EGFR signal transduction pathway, which makes the tumor highly sensitive to the growth-inhibitory effect of the monoclonal antibodies. mAb108 has already been reported to inhibit growth of the human epidermoid cancer KB (22) and the breast cancer MDA-468 (24). In the present study, we have shown that mAb108 can also suppress growth of another human squamous cancer, MH-85, both in vitro and in vivo.

Our results clearly demonstrate that mAb108 alone has inhibitory effects on MH-85 tumor growth in vivo. The antibody is also useful for targeting of cytotoxic drugs or toxins to tumors with overexpressed EGFR. Recently, it has been shown that, when the cytotoxic drug doxorubicin is conjugated to mAb108, doxorubicin is delivered specifically and efficiently to the tumor site, where it can exert its cytotoxic effect on the target tumor cells (43). The conjugates were more effective than either the free drug or antibody given to tumor-bearing nude mice alone. Similarly, it is also reported that immunotoxins made by conjugating monoclonal antibodies against EGFR to toxins such as recombinant ricin A chain (44) or gelonin, a ribosome-inactivating protein (45), are specifically cytotoxic for tumors with increased levels of EGFR expression in vitro and the cytotoxicity is correlated with the numbers of EGFR on target tumors. Thus, monoclonal antibodies to EGFR not only exert anticancer action by themselves but also can be utilized to increase the specificity and efficacy of other anticancer agents.

We have shown that MH-85 has two populations of receptor, with different affinities (dissociation constants of the receptors with high and low affinity are 0.038 and 2.2 nm, respectively). Defize et al. (46) and Bellot et al. (34) have used monoclonal antibodies (including mAb108) to demonstrate that the high affinity binding sites are crucial for EGFR signaling. mAb108, used in the present study, has been shown to interact specifically with high affinity EGFR, which are essential for eliciting several early responses, such as EGFR autophosphorylation, hydrolysis of phosphatidyl inositol, release of calcium from intracellular stores, and elevation of cytoplasmic pH (34).

The MH-85 tumor is associated with several common paraneoplastic syndromes, in the patient from whom the tumor was originally derived as well as in nude mice bearing this tumor. The two most common paraneoplastic syndromes are hypercalcemia and cachexia. In the MH-85-bearing nude mice treated with mAb108 or by removal of the submandibular glands, a major source of EGF, progress of these paraneoplastic syndromes was markedly suppressed. These results raise the possibility that modulation of the EGFR signal transduction pathway is directly responsible for hypercalcemia and cachexia. In fact, TGF-α, which binds to EGF receptors and shares similar biological activities with EGF, has a powerful hypercalcemic action (47), and we have recently found that Chinese hamster ovary cells which have been transfected with the TGF-α gene cause hypercalcemia and cachexia in nude mice. However, we think that this possibility is unlikely in MH-85-bearing nude mice. As we described previously (31), surgical excision of MH-85 resulted in complete recovery from hypercalcemia, leukocytosis, and cachexia. In the present study, inhibition of MH-85 growth by treatment with mAb108 caused impairment of hypercalcemia and cachexia. It seems most likely that development of hypercalcemia and cachexia in MH-85 tumor-bearing animals is intimately dependent on the growth of the tumors.

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