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Glioblastoma-associated circulating monocytes and the release of epidermal growth factor

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Monocytes/macrophages frequently infiltrate malignant gliomas and play a central role in the tumor-associated immune response as they process tumor antigen and present it to T-lymphocytes. Findings have accumulated that peripheral blood monocytes leaving the cerebral circulation become microglial cells and *vice versa* and that monocytes/macrophages may stimulate malignant tumor growth by some unknown mechanism. Most malignant gliomas express growth factor receptors, for example epidermal growth factor receptor (EGFR). The aim of this study was to determine whether peripheral blood monocytes of glioma patients release EGF, the appropriate ligand of gliomacell membrane-bound EGFR.

Long-term cultured peripheral blood monocytes from 14 patients with malignant gliomas were compared to those from 12 controls (seven with nontumorous disease and five healthy individuals). Using an enzyme-linked immunosorbent assay for EGF, the EGF content of cell culture supernatants was determined at Days 7, 21, and 100 of culture. The EGF content (mean ± standard error) of supernatants was 5.9 ± 0.2 pg/ml/10^3 glioma monocytes versus 1.3 ± 0.1 pg/ml/10^3 control monocytes at Day 7 of culture, 22.9 ± 0.8 pg/ml/10^3 glioma monocytes versus 1.8 ± 0.9 pg/ml/10^3 control monocytes at Day 21 of culture, and 23.4 ± 0.7 pg/ml/10^3 glioma monocytes, and below detection levels for control monocytes at Day 100 of culture. Steroid treatment of glioma patients did not influence the EGF release of cultured monocytes. These data indicate that glioblastoma-associated peripheral blood monocytes may be distinct from those of healthy individuals. Moreover, this study indicates that subtypes of glioma-associated peripheral blood monocytes may support immunosuppression and promote growth of malignant glioma by releasing unusually high amounts of EGF.

KEY WORDS • epidermal growth factor • glioma • immune response • macrophage • monocyte • proliferation

EXPRESSION of growth factors, growth factor receptors, and cytokine receptors is seen in most malignant gliomas.[2,4,5,17,22,23,27,31,34,37] One of the most frequently seen and best characterized growth factor receptors in glioblastoma cells is the epidermal growth factor receptor (EGFR). Overproduction of this receptor in glioblastoma cells is due to a translocation or amplification of the *c-erbB2* gene, which encodes for a truncated EGFR protein.[5,34,38] Although such truncated EGFRs may be constitutively active in the absence of ligand binding, EGF binding to normal EGFRs, which in lower numbers can also be found on glioblastoma cells,[17] may corroborate the mitogenic response of these tumor cells.

Whereas several studies[3,4,7,11,13,27,35] have contributed to the characterization of glioma-infiltrating immune cells, little is known about the biochemical properties of circulating immune cells in glioblastoma patients. Findings have accumulated that brain-resident macrophages contribute to glioma infiltration, as well as peripheral blood monocytes that leave the cerebral circulation to become infiltrating microglial cells.[11,13,18] The role that glioma-infiltrating monocytes may play is subject to controversy. On the one hand, they initiate the tumor-associated immune response as they process tumor antigen and present it to T-lymphocytes. On the other hand, tumor-infiltrating monocytes and tumor-associated peripheral blood monocytes have been shown to release growth factors and cytokines that may stimulate the growth of gliomas by binding to growth factor and cytokine receptors on the surface of tumor cells.[3,10,27] Especially when activated, resident and circulating monocytes secrete growth factors related to EGF,[12] transforming growth factor (TGF)–α,[19] platelet-derived growth factor (PDGF),[21] and TGFβ.[26] However, although they are a possible cause of immunosuppression and/or tumor growth stimulation, such properties of monocytes have never been demonstrated in patients with glioblastoma multiforme until now.

To assess the growth factor release potential of circulating immune cells of such patients, the aim of this study was to determine whether glioblastoma-associated peripheral blood monocytes release EGF, the appropriate ligand of glioblastoma cell membrane–bound full-length EGFR. Monocytes/macrophages were chosen because these immune cells regularly infiltrate malignant gliomas.[3,27,35]

Materials and Methods

Patient and Control Groups

The tumor group consisted of 14 adult patients who underwent open or stereotactic surgery for primary glioblastoma (seven patients) and recurrent glioblastoma (seven patients). The grading of the tumors was based on histological criteria according to the World Health Organization classification of brain tumors.[40] There were seven women and seven men, whose mean age at the time of blood sampling and operation was 49.1 years. Seven patients were receiving dexamethasone (3–24 mg/day orally), and seven patients were not receiving steroids at the time of blood sampling. Whereas none of the patients with primary glioblastoma had received anticonvulsant medication, chemotherapy, or radiation therapy, three of seven patients with recurrent glioblastoma were treated with anticonvulsant agents (two with phenytoin and one with carbamazepine), and six of seven patients in that group had received radiation therapy at dose levels of 3800 to 6000 cGy. No patient with glioblastoma was treated with adjuvant chemotherapeutic agents. Tumor samples (nine samples) were obtained during surgery and were immediately frozen. Venous blood samples were taken at 1 to 4 days before surgery to collect peripheral blood monocytes. Peripheral blood monocytes from seven patients with various nontumorous diseases (brain infarction, subarachnoid hemorrhage, acute subdural hematoma, cervical disk herniation) and from five healthy donors served as controls. The control group consisted of six women and six men with a mean age of 41 years. Six of the seven control patients with nontumorous diseases were receiving dexamethasone (4.5–32 mg/day orally or intravenously), whereas none of the five healthy donors received any medication. All individuals receiving dexamethasone medication were administered histamine H_2-blocking agents (ranitidine or famotidine) to prevent gastrointestinal ulcers. The composition of the glioblastoma group and the control group including all being given therapeutic agents was chosen to exclude effects of age, sex, dexamethasone therapy, histamine H_2-receptor blocking medication, anticonvulsant medication, or radiation therapy on the EGF production of circulating monocytes.

Immunohistochemical Techniques

To analyze EGFR immunoreactivity and monocyte/macrophage (Leu-M3) immunoreactivity of the glioblastoma specimens, frozen sections were immunostained by a sequential incubation with mouse anti–human EGFR or Leu-M3 monoclonal antibodies (MAbs), followed by peroxidase-conjugated rabbit anti–mouse immunoglobulin G (IgG) MAbs. Localization of immunoreactivity was detected using a peroxidase reaction with 3,3′-diaminobenzidine tetrahydrochloride as the chromogen.

Separation of Blood and Cell Culture of Circulating Monocytes

Differential white blood cell counts were performed with May–Grünwald/Giemsa stains. Simultaneously, 24 ml of citrated venous blood from each subject, mixed with the same amount of Hanks' balanced salt solution (HBSS) underwent density gradient centrifugation over Ficoll. Immediately after centrifugation the remaining fresh plasma was diluted 1:1 with HBSS, frozen, and stored at −80°C to determine plasma levels of EGF.

Using cell culture dishes, 5×10^5 monocytes from each participant were cultured in RPMI-1640 supplemented with glutamine, penicillin, streptomycin, and 5% fetal calf serum (endotoxin content < 2.5 U/ml). The cultures were kept in an incubator at 37°C in an atmosphere of 5% CO_2 in room air. At 6- to 7-day intervals, fresh culture medium was added to the monocyte cultures, and in each of them the number of adherent vital cells within five randomized optical fields was counted with a phase-contrast inverted microscope.

Phenotype Analysis and Determination of EGF Release

The immunophenotype of cultured cells was determined by incubating the cells in the dark at 4°C with fluorescein isothiocyanate-conjugated mouse anti–human Leu-M3 MAbs at saturating amounts. After 20 minutes the cells were washed with ice-cold phosphate-buffered saline and observed in a semidark chamber with a fluorescence microscope. The C-6 rat glioma cell line served as a nonmonocyte control cell line.

The culture supernatants were decanted, immediately frozen and stored at −80°C at Days 7, 21, and 100. A sample of each supernatant was quantitatively analyzed for soluble CD14 (sCD14), a surface antigen restricted to monocytes and immature macrophages. A commercially available sCD14-specific enzyme-linked immunosorbent assay (ELISA) was used. In addition, all plasma samples diluted 1:1 with HBSS as well as samples of the culture supernatants collected at Days 7, 21, and 100 of culture were analyzed for EGF. An EGF-specific ELISA was used.

Statistical Analysis

Mean values and standard errors (SE) of sCD14 and EGF production per milliliter adjusted to 10^3 cells were calculated for the following experimental groups, respectively: all glioblastomas, glioblastomas with steroid therapy, glioblastomas without steroid therapy, and controls. Differences between the groups were evaluated using the Kruskal–Wallis test with multiple comparisons on ranks of several independent samples.[32]

Sources of Supplies and Equipment

Mouse anti–human EGFR MAbs and EGF-specific ELISA were obtained from Dianova, Hamburg, Germany. Mouse anti–human Leu-3 MAbs were obtained from Becton-Dickinson GmbH, Heidelberg, Germany, and rabbit anti–mouse IgG MAbs from Sigma, Deisenhofen, Germany. The phase-contrast inverted microscope was purchased from Leitz, Wetzlar, Germany, and the fluorescence microscope from Zeiss, Oberkochen, Germany. The sCD14-specific ELISA was obtained from IBL, Hamburg, Germany.

Results

Immunophenotype of Cultured Mononuclear Cells

The supernatants of glioblastoma monocyte and control cultures contained stable amounts of 0.38 ± 0.04 ng/ml/10^3 to 0.55 ± 0.04 ng/ml/10^3 monocytes (mean ± SE) of sCD14 monocyte surface antigen throughout the time of culture. Immunostaining of cell culture samples with Leu-M3 antibodies for the CD14 monocyte/macrophage antigen and analysis of sCD14 in the culture supernatants revealed that the cultured mononuclear cells were representative of the monocyte/macrophage lineage.

Epidermal Growth Factor Release of Cultured Monocytes

At Day 7 the EGF content of glioblastoma-associated monocyte culture supernatants was 5.9 ± 0.2 pg/ml/10^3 monocytes (mean ± SE), at Day 21 of culture it was 22.9 ± 0.8 pg/ml/10^3 monocytes, and at Day 100 it reached 23.4 ± 0.7 pg/ml/10^3 monocytes (Fig. 1 *left*).

At Day 7 the EGF content of control monocyte culture supernatants was only 1.3 ± 0.1 pg/ml/10^3 monocytes, and at Day 21 the EGF content in control supernatants was 1.8 ± 0.9 pg/ml/10^3 monocytes (Fig. 1 *left*). However, at Day 100 of culture, 57% of the glioblastoma-associated monocyte cultures contained viable cells growing in clusterlike formations, whereas only one control monocyte culture retained a low number of monocytes. For this reason, the EGF content of control monocyte culture supernatants was deemed to be below the level of detection at Day 100.

Statistical analysis showed significant differences between the EGF content of monocyte supernatants derived from patients with glioblastoma and those derived from

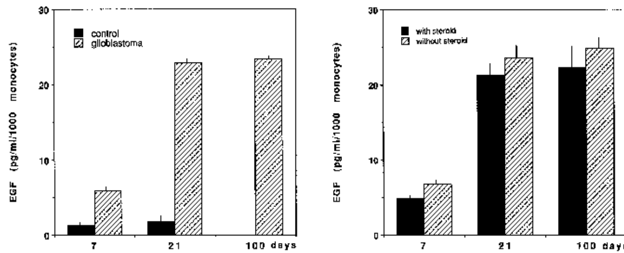

FIG. 1. Graphs showing the epidermal growth factor (EGF) content per milliliter per 10^3 cells at Days 7, 21, and 100 in culture supernatants of peripheral blood monocytes. *Left:* Graph showing the monocytes derived from 12 controls and 14 patients with glioblastoma. At Days 7 and 21, the EGF content of glioblastoma monocyte culture supernatants was significantly higher than in control monocyte cultures (Day 7, $p < 0.01$; Day 21, $p < 0.001$; Kruskal–Wallis test). At Day 100, the EGF content of the only remaining control monocyte culture was below the level of detection. *Right:* Graph showing the monocytes derived from glioblastoma patients with (seven patients) and without (seven patients) dexamethasone treatment at the time of blood sampling. Statistical analysis (Kruskal–Wallis test) revealed the differences between groups to be nonsignificant, although the EGF content of supernatants of glioblastoma-associated monocyte cultures derived from patients with dexamethasone treatment tended toward slightly lower values.

nontumorous controls at Day 7 ($p < 0.01$, Kruskal–Wallis test) and at Day 21 ($p < 0.001$, Kruskal–Wallis).

Steroid treatment in patients with glioblastoma at the time of blood sampling did not significantly affect the EGF release of glioblastoma-associated peripheral blood monocytes in culture (Fig. 1 *right*). Nevertheless, a tendency toward somewhat lower EGF levels was seen in cultures derived from patients treated with steroids. The EGF content of monocyte culture supernatants derived from glioblastoma patients receiving steroid treatment was 5 ± 0.2 pg/ml/10^3 monocytes (mean $\pm$ SE) at Day 7, 21.8 ± 1.5 pg/ml/10^3 monocytes at Day 21, and 22.4 ± 2.7 pg/ml/10^3 monocytes at Day 100, respectively. The EGF content of monocyte culture supernatants derived from glioblastoma patients not receiving steroid treatment was 6.6 ± 0.4 pg/ml/10^3 monocytes at Day 7, 23.8 ± 1.5 pg/ml/10^3 monocytes at Day 21, and 24.8 ± 1.4 pg/ml/10^3 monocytes at Day 100, respectively.

Plasma Levels of EGF

The EGF plasma levels of all samples, whether tumor or control derived, were below the level of detection of the ELISA technique used, probably because of the diluting effect of the plasma water itself or of the HBSS added to the blood before the density gradient centrifugation.

Monocyte Marker and EGFR Immunoreactivity in Tumor Specimens

All glioblastoma specimens were immunoreactive for EGFR and Leu-M3 (Fig. 2), which is a marker for monocytes and immature macrophages. The EGFR-positive cells as well as the Leu-M3–positive cells were found to be diffusely distributed within the tumor, with a propensity of Leu-M3–positive cells to circumscribe the perivascular spaces (Fig. 2a) and a tendency of EGFR-positive cells to be spread in patchy formations. The ratio of EGFR-positive cells in the various tumor specimens varied from approximately 10% to more than 90% (Fig. 2b). Similarly, the ratio of Leu-M3–positive cells in the examined glioblastomas varied from 15% to approximately 40%. The number of EGFR-positive tumor cells did not correlate with the number of Leu-M3 monocyte lineage–derived tumor infiltrating cells. The intensity of EGFR immunoreactivity and that of Leu-M3 immunoreactivity in the tumor tissue was unaffected by steroid treatment in the respective patients with glioblastoma.

Discussion

Immunosuppression by EGF

Our data demonstrate that glioblastoma-associated peripheral blood monocytes release substantially larger amounts of EGF even over a long period of time (100 days) than do monocytes from healthy individuals or from patients with nontumorous diseases. In both the glioblastoma and the nontumor group the EGF production of cultured monocytes was not influenced by medication, especially not by dexamethasone therapy.

In a recently published paper[10] we could demonstrate that the long-term survival of monocytes derived from patients with glioblastomas coincides with excess release of interleukin 1β (IL-1β) and that subpopulations of monocyte cells may be continuously activated through autocrine stimulatory loops via the IL-1β–IL-1β receptor pathway in such monocyte cultures. In addition, the long-term cell survival of glioblastoma-associated monocytes may be enhanced as well by growth factors such as EGF, because EGF has been shown to have an effect on immune cells. Because EGF elicits immunosuppressive activity,[16,25] and although immunosuppression may not directly cause malignant transformation of glial cells *in vivo*, it is considered to facilitate tumor growth.[30] Earlier studies have demonstrated that EGF, on the one hand, potentiates the antigen-presenting mechanism of monocytes and lymphocytes,[1] but on the other hand induces a substantial immunosuppression. For instance, EGF exerts a short-term as well as a long-term downregulation of helper T-cell function[25] and a stimulation of cortisol production in the adrenal glands.[28] In this regard, it should be mentioned that in our study, in patients with glioblastomas who were receiving steroid treatment at the time of blood sampling the subsequent EGF production of circulating monocytes *in vitro* was not significantly influenced.

Recently, Evans, *et al.*,[8] reported that tumor-derived products induce growth factor gene expression in murine macrophages, and that there are differences between tumor-induced and bacterial endotoxin-induced gene expression. If these experimental data hold true *in vivo* in glioblastoma patients, the evolution and increase in the number of aberrant immunosuppressive glioma-associated monocytes/macrophages, whether tumor-infiltrating or circulating, would be a selective effect of the glioblastoma itself. Several authors have shown that, on activation, monocytes or macrophages release growth factors and express growth factor-related protooncogenes that are involved in malignant transformation and proliferation of

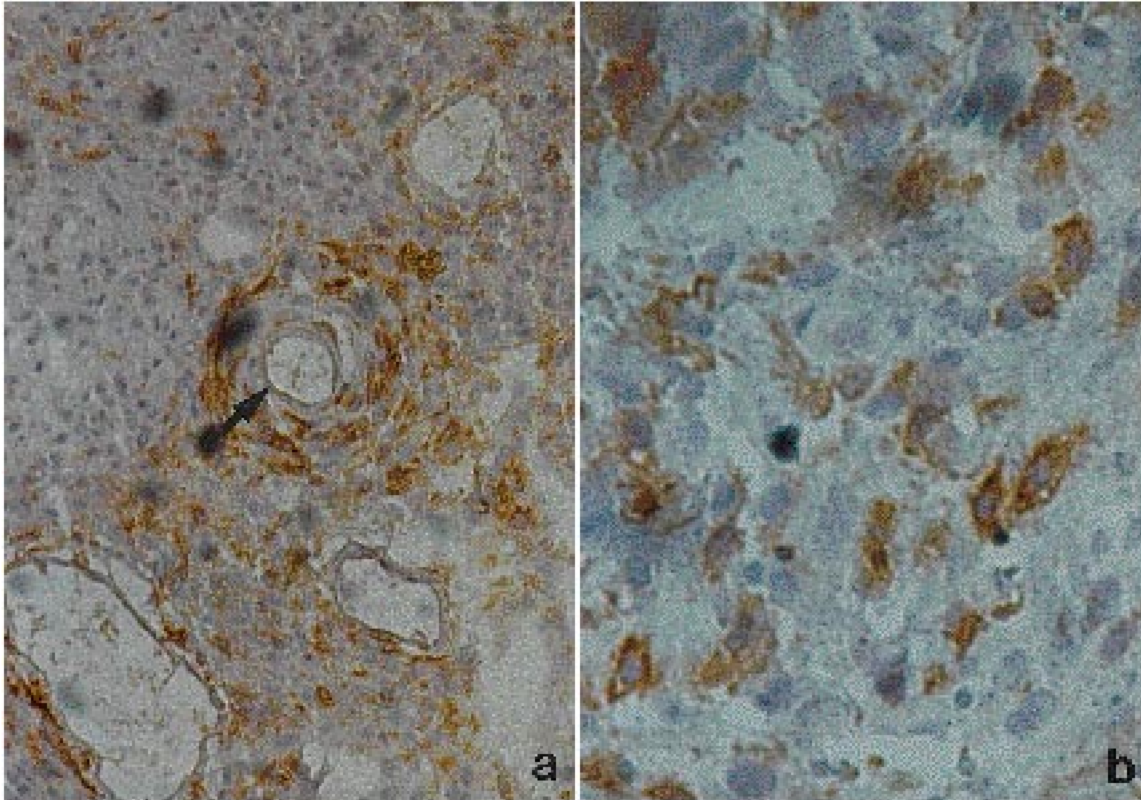

FIG. 2. Photomicrographs showing immunoreactivity in glioblastoma specimens. a: Photomicrograph showing strong monocyte/macrophage marker (Leu-M3) immunoreactivity in a glioblastoma specimen. Approximately 25% of the cells were Leu-M3–positive and were therefore identified as tumor-infiltrating monocytes or immature macrophages. The Leu-M3–positive cells showed a tendency to be localized more frequently in the perivascular spaces. *Arrow* indicates a blood vessel. Original magnification × 100. b: Photomicrograph showing strong epidermal growth factor receptor (EGFR) immunoreactivity in a glioblastoma specimen. Approximately 20% of the cells were EGFR positive. Most of these EGFR-positive cells were arranged in patchy formations. Original magnification × 250.

gliomas, such as TGFα,[19] TGFβ,[26] EGF,[12] c-*sis* proto-oncogene, and PDGF-like polypeptides.[21]

This is the first study demonstrating that glioblastoma-associated circulating monocytes release EGF. The large amount of EGF released from glioblastoma-associated peripheral blood monocytes may be a constitutive property of these cells, because the production of EGF was maintained up to 100 days after the cells were removed from the circulation and from the influence of drugs or tumor-derived substances in the patients' serum. These data and the fact that increased EGF production was found in all tumor-associated monocytes, whether derived from patients with primary or with recurrent glioblastomas, corroborate the assumption that subtypes of glioblastoma-associated monocytes may be aberrant immune cells, or that subsets of glioblastoma-associated circulating monocytes are at least maximally activated cells that release large amounts of EGF, which acts as a potential immunosuppressive agent in glioblastoma patients. Although not directly confirmed in this study, it seems that monocyte cultures derived from glioblastoma patients may contain specific subpopulations of monocytes in which the gene for EGF is continuously activated. The underlying mechanism for this continuous gene activation remains unclear. However, it is well known from previous studies[12,19,21,26] that monocytes and macrophages retain genes encoding for a number of growth factors, which are released continuously or on activation of monocytes/macrophages. More detailed studies are necessary to characterize glioblastoma-associated monocytes further, whether resident or circulating. In this regard, it would also be interesting to investigate whether these possibly aberrant circulating monocytes are on their way to the tumor or have just left the tumor to reinvade immunocompetent organs.

Stimulation of Glial Cell Proliferation by EGF

In addition to its potential immunosuppressive activity in glioma patients, EGF is considered to be a stimulator of normal and neoplastic glial cell proliferation. A 6-kD polypeptide composed of 53 amino acids, EGF is synthesized by a number of different nonneoplastic cells.[30,39] Transforming growth factor–α, a 5.6-kD polypeptide consisting of 50 amino acids and sharing sequence homology with EGF,[20] is produced by various transformed and neoplastic cells.[24,29,33] Transforming growth factor–α competes with EGF for binding to the EGFR. The stimulatory and inhibitory effects of EGF on cellular proliferation and differentiation depend on the target cell type, its state

of differentiation,[14,30] the number of EGFRs on the target cell, and the presence of other growth factors, such as TGFα, competing with EGF for binding to the same cell surface receptor[33] or such as IL-1, which has been shown to potentiate the EGF-induced proliferative response.[15]

The addition of EGF to normal glial cells[36] or malignant glial cell lines[23] elicits responses that are associated with neoplastic transformation. In other words, EGF directly elicits transformation-associated phenotypes in certain target cells.[14,30] Our study confirms findings that glioblastomas retain large numbers of EGFR-positive cells[2,17,22,27,34,37] and large numbers of tumor-infiltrating monocytes as well.[3,27,35] Thus, whereas EGF seems to play a physiological role in the normal central nervous system,[9] the increased production of EGF by glioblastoma-associated monocytes even over a long period of time (100 days in culture) must be hypothesized to stimulate the proliferation of glioblastoma cells, provided that these monocytes retain the capacity to infiltrate into the gliomas. However, future studies will have to confirm this suspected property of glioblastoma-associated circulating monocytes. If this hypothesis holds true, such large amounts of EGF released from tumor-infiltrating or circulating monocytes may further contribute to glioma growth by neoplastic transformation of neighboring normal glial cells, because it has been shown that EGF added to normal glial cells induces a partial loss of density-dependent inhibition of growth,[36] a characteristic of malignant transformation, which is perhaps due to the decrease of cell-surface fibronectin on EGF-stimulated cells.[6]

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