

Gastrin Significantly Modifies the Migratory Abilities of Experimental Glioma Cells

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SUMMARY: Malignant astrocytic tumors are characterized by the pronounced and diffuse migration of tumor astrocytes into the brain parenchyma. The present study shows that gastrin is a brain neuropeptide that is able to significantly modulate astrocytic tumor migration at both invasion and motility levels. In the matter of invasion, gastrin severely reduces the *in vitro* invasive abilities of C6 rat glioma, 9L rat gliosarcoma, and U373 human glioma cells in a collagen matrix. *In vitro*, gastrin also markedly modifies the motility features in both C6 and U373 cells, at least partly through a decrease in the expression of the RhoA small GTPase, and so brings about some dramatic modifications to the organization in the actin cytoskeleton. The *in vitro* preincubation of C6 tumor cells with gastrin significantly increases the life spans of rats stereotactically implanted with these cells as compared with the survival periods of rats implanted with gastrin-untreated C6 cells. As suggested by our *in vitro* experiments, these effects, observed *in vivo* cannot relate to only the gastrin-induced decrease in tumor astrocyte migratory abilities. Indeed, gastrin also induces immunomodulatory effects, because we observed a marked gastrin-induced recruitment of lymphocytes into C6 gliomas and 9L gliosarcomas. These data all suggest that gastrin can act as an endogenous modulator of glioma progression. (*Lab Invest* 2002, 82:1241–1252).

Brain tumors constitute approximately 10% of all solid tumors in adults and between 20% and 30% in children (Burger and Scheithauer, 1994; Kleihues and Cavenee, 2000). Approximately one-third are diffuse astrocytic tumors (astrogliomas) associated with dismal prognoses because of their pronounced ability to diffusely invade the brain parenchyma. Their level of malignancy increases from astrocytomas (World Health Organization/WHO Grade II) to anaplastic astrocytomas (WHO Grade III) and glioblastomas (WHO Grade IV) (Kleihues and Cavenee, 2000). The invasive abilities of individual tumor astrocytes depend mainly on their migratory potential (Chintala and Rao, 1996; Giese et al, 1994), and part of this potential depends on the organization of the actin cytoskeleton (Cramer and Mitchison, 1993; Lauffenburger and Horwitz, 1996).

Whereas gastrin is known to significantly modulate both the proliferation levels of many tumor types (Rehfeld and van Solinge, 1994) and the migration levels of various cell types of the immune system (de la Fuente et al, 1997, 1998), we were the first to show that gastrin is able to modulate both proliferation (Camby et al, 1996) and migration (De Hauwer et al, 1998) in human tumor astrocytes. These data could relate to the fact that cholecystokinin (CCK) and CCK-related peptides (gastrin) constitute the most abundant peptide system in the brain (Rehfeld and van Solinge, 1994), with gastrin-induced effects mediated through the CCK_B receptor. This receptor is the predominant CCK/gastrin receptor of the central nervous system (CNS), whereas the CCK_A receptor, which is a thousand times more specific to sulfated CCK-8 than to gastrin, is predominant outside of the CNS (Rehfeld and van Solinge, 1994). Normal astrocytes possess significant amounts of CCK_B receptors (Hosli and Hosli, 1994) and might be a major primary target for CCK/gastrin peptides in the CNS (Muller et al, 1997).

Although during the last 3 years we have accumulated a large set of data indicating that gastrin targets several genes directly involved in the proliferation and migration features of tumor astrocytes (see "Discussion"), neither we nor anyone else has investigated the potential role played by gastrin in *in vivo* glioma progression. In our study, we therefore made use of the C6 rat and the U373 human astroglioma models (and to a lesser extent the 9L rat gliosarcoma) to

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investigate gastrin-induced effects in vitro with respect to invasion and motility. For our in vivo experiments, we made use of stereotactic implantations of C6 and 9L tumor cells into rat brains. The development of the C6 gliomas and 9L gliosarcomas in the brain was monitored by means of nuclear magnetic resonance (NMR) imaging, and once a rat began to lose weight, a surgical procedure was applied to remove the bulk of the C6 and 9L tumor. Subcutaneously implanted micropumps were then used to deliver gastrin into the C6 and 9L tumor resection cavities. C6 cells were also preincubated in vitro with various concentrations of gastrin before their stereotactic implantation into the rats' brains to study the direct effects of gastrin on in vivo C6 glioma progression. The potential gastrin-induced immunomodulatory counter-effects on C6 gliomas and 9L gliosarcomas stereotactically implanted into the rats' brains were also investigated, because gastrin has a chemotactic influence on a large set of cells of the immune system (de la Fuente et al, 1997, 1998). It should be emphasized that the C6 model suffers from some limitations because it is allogenic and so induces significant host-mediated immunogenic responses (Parsa et al, 2000). We therefore added to our in vivo experiments the 9L gliosarcoma model as an internal

methodologic control because this model is syngeneic and therefore weakly immunogenic (Parsa et al, 2000). The presence of mRNAs of cholecystokinin (CCK_A) and gastrin (CCK_B) receptors was assayed on the U373, C6, and 9L models by means of RT-PCR techniques. The presence of CCK_C in human tumors, a third CCK /gastrin receptor, has already been described (Baldwin, 1994). We therefore also assayed the presence of mRNAs for CCK_C receptors in the U373 human glioma model. Gastrin production in C6, 9L, and U373 cells was evidenced at both RNA and protein levels by means of the quantitative RT-PCR and ELISA assay respectively.

Results

Identification of CCK_A , CCK_B , and CCK_C Receptors and Gastrin RNAs

Although U373 human glioma cells exhibit RNAs of CCK_C receptors (Fig. 1B), we failed to evidence RNAs of either CCK_B (Fig. 1A) or CCK_A (data not shown) receptors. Although C6 tumor cells exhibit RNAs of CCK_B (Fig. 1D), but not of CCK_A receptors (Fig. 1C), the reverse was observed with respect to the 9L tumor cells (Fig. 1, C and D).

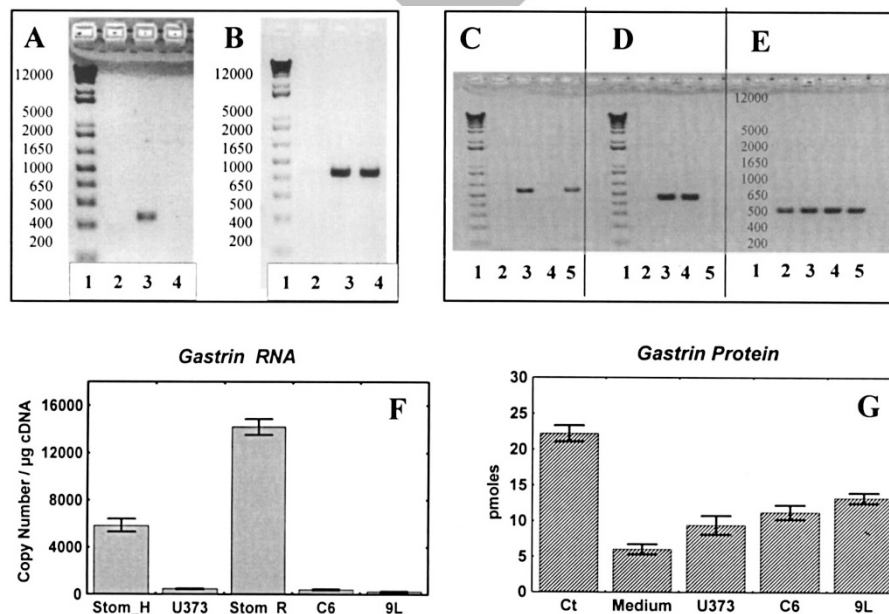


Figure 1.

A, Analysis of the expression of cholecystokinin (CCK_B) receptor RNA in U373 human gliomas (Lane 4) and human Jurkat cells chosen as positive control (Lane 3). Lane 2 corresponds to negative control (H_2O), and Lane 1 to the molecular weight markers. B, Analysis of the expression of CCK_C receptor RNA in U373 human gliomas (Lane 4) and human LoVo colon cancer cells chosen as positive control (Lane 3). C, Analysis of the expression of CCK_A receptor RNA in C6 (Lane 4) and 9L (Lane 5) rat tumor cells. Lane 3 corresponds to a positive control, normal pancreatic rat tissue. Lane 2 corresponds to negative control (H_2O), and Lane 1 to the molecular weight markers. D, Analysis of the expression of CCK_B receptor RNA in C6 (Lane 4) and 9L (Lane 5) rat tumor cells. Lane 3 corresponds to a positive control, normal rat brain tissue. Lane 2 corresponds to negative control (H_2O) and Lane 1 to the molecular weight markers. E, Analysis of the expression of β -actin RNA in normal pancreatic rat tissue (Lane 2), 9L rat tumor cells (Lane 3), normal rat brain tissue (Lane 4), and C6 rat tumor cells (Lane 5). Lane 1 corresponds to negative control. The same characterization of β -actin RNA expression was also performed for the U373 tumor cells and their corresponding normal positive controls, ie, Jurkat and LoVo cells (not shown). F, Quantitative RT-PCR analysis of gastrin RNA in U373, C6, and 9L tumor cells. Samples of human (Stom_H) and rat (Stom_R) stomach tissue were used as positive controls. Four independent experiments were carried out. The data are presented as mean \pm SEM values. G, Determination of gastrin protein amounts in U373, C6, and 9L tumor cells by means of an ELISA assay. Ct represents a positive control condition in which 10^{-10} M gastrin was added to a serum-free medium and assayed 24 hours later. Amounts of gastrin were also assayed in culture media supplemented with 10% fetal calf serum (medium), and in the culture media of C6, 9L, and U373 tumor cells 24 hours after their plating (5,000 tumor cells per milliliter of medium) into the culture media. Gastrin was assayed in the culture media free of tumor cells.

We did not investigate the RNAs for the CCK_C receptor in rat tumor cells because, to date, no CCK_C receptor has been cloned in a rat. C6, 9L, and U373 cells produce weak but nevertheless significant amounts of gastrin. We evidenced this feature at both RNA (Fig. 1F) and protein (Fig. 1G) levels.

In Vitro Gastrin-Induced Modifications to C6, 9L, and U373 Cell Migration (Invasion and Motility) Features

We characterized the gastrin-induced effects on C6, 9L, and U373 cell invasion features by quantifying the percentage of C6, 9L, and U373 cells able to invade a collagen matrix. The data show that a 24-hour gastrin pulse induced a marked dose-dependent decrease in the invasiveness of the three experimental models (Fig. 2, A–C). Although the invasion by tumor astrocytes or gliosarcoma cells of type I collagen gels may have some relevance to their *in vivo* growth, it cannot be assumed that *in vitro* collagen invasion by tumor astrocytes and/or gliosarcoma cells is relevant in the case of brain invasion by glioma or gliosarcoma cells in which type I collagen is absent. We are setting up new tests involving laminins, fibronectins, and vitronectins in place of type I collagen.

Motility refers to a cell's capacity for locomotion. We used computer-assisted phase-contrast microscopy to quantify the gastrin-induced effects on C6 (Fig. 2D) and U373 (Fig. 2E) cell motility levels. Although gastrin induced a dose-dependent decrease in the U373 cell motility level (Fig. 2E), it slightly increased the C6 cell motility level when added to the culture media at 10 nM (Fig. 2D). This effect on C6 cells was no longer statistically significant at 0.1 nM and 1,000 nM (Fig. 2D). Gastrin had no statistically significant influence on the motility levels of the 9L cells (not shown).

L-365,260, a specific CCK_B receptor antagonist, significantly decreased the level of C6 motility when added to the culture media at 10 or 1,000 nM for at least 24 hours (Fig. 2D). This feature relates to the fact that C6 cells secrete gastrin, as we observed at both gastrin mRNA (by means of RT-PCR) and protein (by means of Western blotting analyses) levels (Fig. 1, F and G). Gastrin may therefore play an autocrine role in tumor astrocyte migration.

A marked decrease in C6 cell motility level was observed after 24 hours of videomicroscopy observation when the L-365,260 compound was added to the culture media at the same time as gastrin (both at 10 nM) (Fig. 2D). All of these features therefore suggest that the gastrin-induced increase in C6 cell motility occurs via the CCK_B receptor. Indeed, when the CCK_B receptor activity was blocked by means of the L-365,260 compound, gastrin induced a decrease in C6 cell motility, a phenomenon that may have taken place via the CCK_C receptor as strongly suggested by the data obtained for the U373 model (Fig. 2E).

When gastrin was added 1 to 3 hours before its antagonist, the activity of the CCK_B receptor was not blocked when 10 nM of gastrin was added to the culture media of the C6 cells. Consequently, gastrin

was no longer able to significantly inhibit C6 cell motility through the CCK_C receptors (Fig. 2D).

Gastrin-Induced Modifications to the C6 Cell Cytoskeleton and the RhoA Protein Level

One of the most important biologic features in cell motility is the question of actin polymerization/depolymerization dynamics. Although fibrillary actin can be demonstrated in cells by means of a phalloidin derivative (phalloidin) conjugated with a green fluorochrome (Fig. 3, A and B), globular actin can be shown by means of DNaseI conjugated with a red fluorochrome (Fig. 3, A and B). Specific fibrillary/globular actin ratios are related to maximum cell motility features (Lauffenburger and Horwitz, 1996). Culturing C6 cells for 24 hours with 10⁻⁸ M gastrin markedly reduced the amount of fibrillary actin (Fig. 3B) as compared with what occurred when these cells were cultured in the absence of gastrin (Fig. 3A). The quantitative data obtained by means of computer-assisted fluorescence microscopy show that gastrin markedly reduced the amount of fibrillary actin (in favor of globular actin) in a dose-dependent manner (Fig. 3C). These data should be seen in the light of those reported for the gastrin-induced modifications to C6 (Fig. 2D) and U373 (Fig. 2E) cell motility features.

Quantitative Western blotting indicated that gastrin significantly decreased the RhoA expression (a potent modulator of actin polymerization/depolymerization dynamics) (Fig. 3D). These data suggest that the gastrin-induced decrease in the amount of fibrillary actin observed *in vitro* (Fig. 3C) might relate to the gastrin-induced decrease in RhoA expression evidenced here.

Gastrin-Induced Modifications to the Survival Periods of C6 Glioma-Grafted Rats

To investigate whether gastrin is able to significantly modify the survival of rats intracranially grafted with the C6 model, we preincubated *in vitro* C6 cells with various concentrations of gastrin before stereotactically grafting these gastrin-treated cells into the rat brains. The fact of preincubating C6 cells *in vitro* with gastrin and then grafting them *in vivo* into rat brains enabled us to investigate whether gastrin-induced modifications to migration features (as detailed in Fig. 2), and consequently to the organization of the actin cytoskeleton (as detailed in Fig. 3), could influence the survival of rats stereotactically implanted with these gastrin-treated tumor astrocytes. The data in Figure 4A clearly indicate that the 24-hour *in vitro* gastrin pretreatment of the C6 cells before their *in vivo* brain implantation significantly increased the rats' survival at the 10⁻⁸ M and 10⁻¹⁰ M doses, but not at the 10⁻⁶ M or the 10⁻¹² M doses. These data thus suggest that the C6 cells pretreated *in vitro* with gastrin partially lost their biologic aggressiveness when subsequently grafted *in vivo* into the rats' brains. As checked by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl

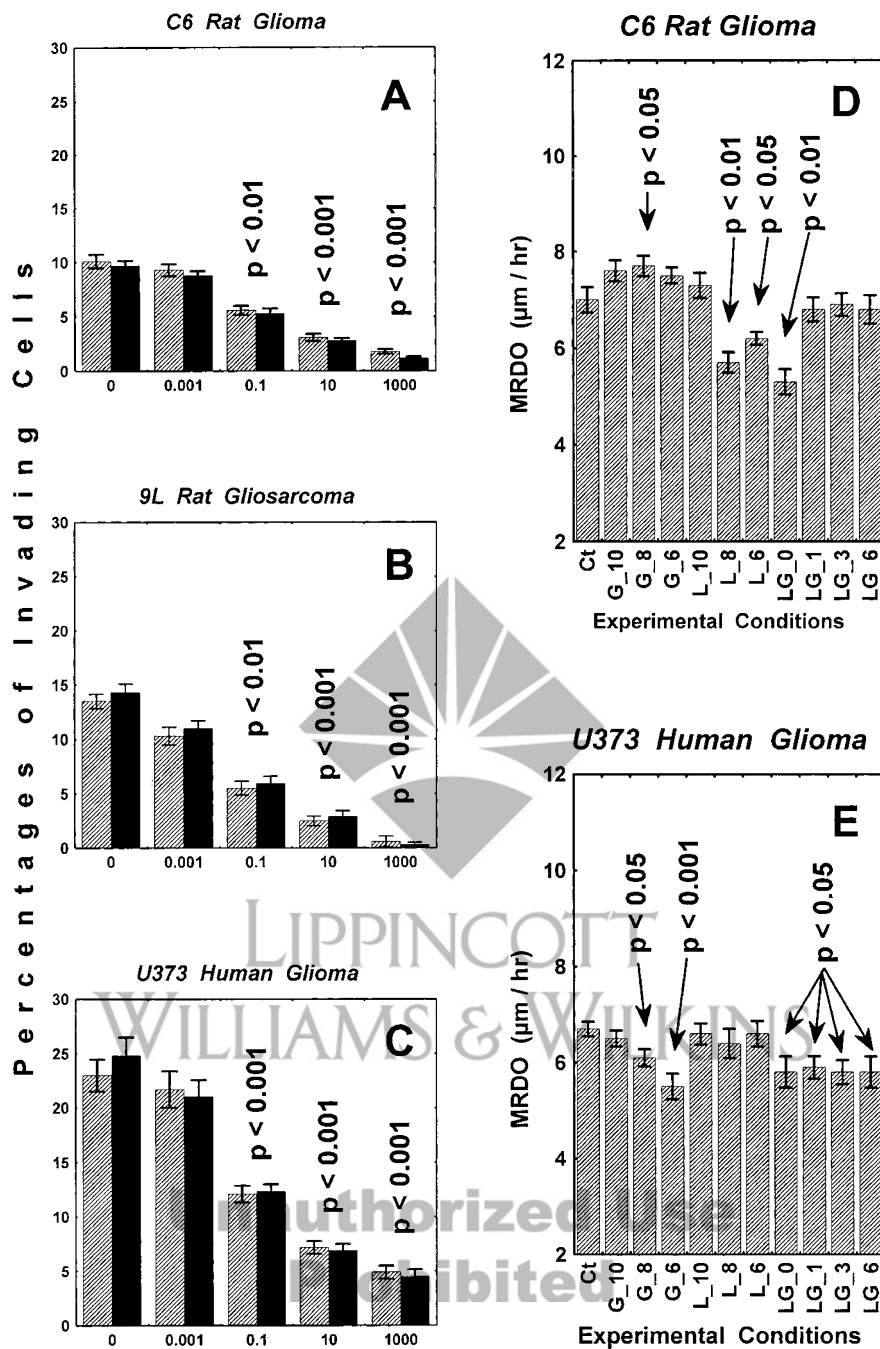


Figure 2.

A to C, Characterization of the influence of gastrin on the invasive potential of the C6 rat glioma (A), the 9L rat gliosarcoma (B), and the U373 human glioma (C) models. The assay consisted of a 90% collagen I / 10% collagen III matrix on which the cells were plated, and the numbers (expressed as percentages) of cells succeeding in invading this matrix were recorded. Two independent sets of experiments (black and shaded bars) were performed. The tumor cells were cultured for 24 hours with four different doses of gastrin before being plated on the collagen matrix. The thick bars represent the mean values and the lines above the bars represent the standard errors. Statistical analyses were performed by means of the Dunn procedure. D and E, Characterization of the influence of gastrin (G) and L-365,260 (L, a specific CCK_b receptor antagonist) on the motility behavior (determined by the maximum relative distance from the point of origin [MRDO] variable) of the C6 (D) and U373 (E) tumor cells after 24 hours of quantitative videomicroscopy. The cells' motility behavior was evaluated for those left untreated (control = Ct) and those treated with 0.1 (G_10), 10 (G_8), and 1,000 (G_6) nM of gastrin, with 0.1 (L_10), 10 (L_8), and 1,000 (L_6) nM L-365,260, or with both compounds (LG conditions). In this last set of experimental conditions, gastrin was always used at a 10 nM concentration and the L-365,260 compound was added at a 10 nM concentration, either at the same time as the gastrin (LG_0), or 1, 3, or 6 hours after the addition of gastrin (LG_1, LG_3, LG_6, respectively). Triplicate analyses were performed, with a minimum of 844 and a maximum of 1,008 cells analyzed in each experimental condition. The data are presented as means ± their standard errors. Statistical analyses were performed by means of the Dunn procedure.

tetrazolium bromide (MTT) colorimetric assay, the levels of C6 tumor cell viability were similar ($p > 0.05$) in each control and gastrin-treated batch of C6 tumor

cells grafted into the rats' brains (not shown). These gastrin-induced effects, illustrated in Figure 4A, were highly reproducible; similar data were obtained in a

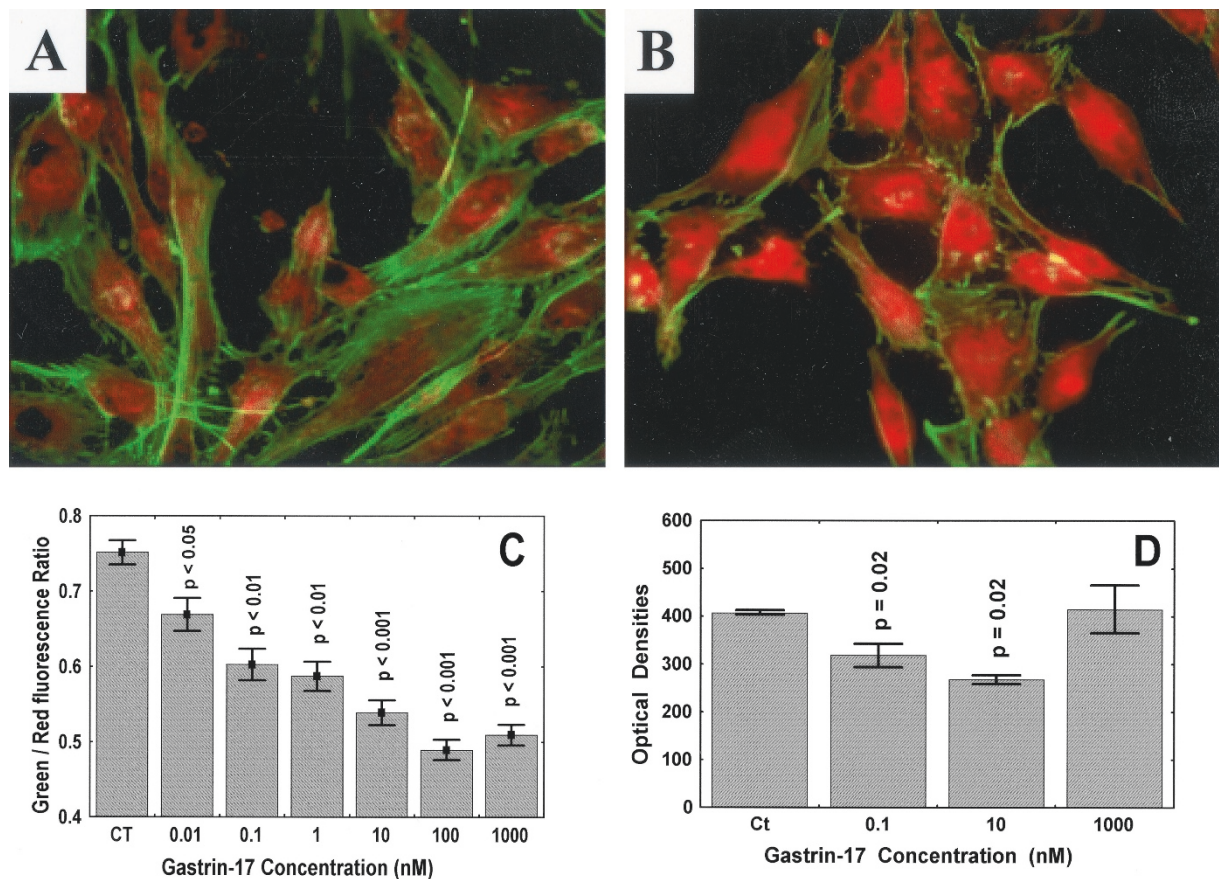


Figure 3.

A and B, Appearance of fibrillary (green fluorescence) as opposed to globular (red fluorescence) actin in C6 tumor astrocytes cultured for 24 hours, with (B) and without (A) 10^{-8} M gastrin. C, Characterization of the influence of gastrin assayed at six concentrations (CT = control) with respect to the relative amounts of fibrillary (green fluorescence in A and B) to globular (red fluorescence in A and C) actin quantitatively determined by means of computer-assisted fluorescence microscopy. The data are reported as means \pm their standard errors. Statistical analyses were performed by means of the Dunn procedure. D, Influence of gastrin on the expression of the RhoA protein (quantitatively determined by means of Western blotting analyses) compared with control (CT). C6 cells were incubated for 24 hours in the presence (or the absence in control) of the various gastrin concentrations before proteins were extracted for Western blotting analysis. The data are presented as mean \pm SEM values. Four independent experiments were carried out.

second experiment performed 6 months later (not shown).

Although direct gastrin-induced modifications to glioma progression are strongly suggested by the data detailed in Figures 2, 3, and 4, it is nevertheless surprising that the in vitro gastrin-pretreatment of the C6 cells could alone be responsible for such significant increases in the rats' survival (Fig. 4A). Indeed, because the C6 glioma model is allogenic (Parsa et al, 2000), some gastrin-induced immune-mediated effects must also be considered in addition to the suspected gastrin-induced effects at migration level. We therefore conducted a new set of experiments in which we investigated whether gastrin was associated with any immunomodulatory effects. For this purpose we implanted C6 tumor cells by means of stereotactic procedures and surgically removed the glioma bulk some weeks later, as detailed in the "Materials and Methods." The data illustrated in Figure 4B show that gastrin delivery carried out on the same day as surgery (*open circles*) seems to have triggered a weak, but nevertheless statistically significant, benefit in terms of survival in the case of approximately 10 of the 18 rats

included in this experimental group. This effect must be compared with the one observed in the group of 18 rats that underwent surgery without subsequent gastrin delivery (*filled circles*). This benefit occurred mainly during the 2 weeks after the end of the gastrin delivery, that is, from Day 7 to Day 21 after surgery. No benefit from any treatment (ie, surgery alone or surgery and gastrin release) was observed in the case of the 9L gliosarcoma (not shown). All of the animals grafted with the 9L gliosarcoma died between 2 and 7 days after they had begun to lose weight (not shown).

Two long-term survival rats (grafted with the C6 glioma) that underwent surgery but no subsequent gastrin delivery (*asterisks*; Fig. 4B) and six in the surgery-plus-gastrin-delivery group (*open circles*; Fig. 4B) were killed 60 days after surgery for the histopathologic examination of their brains. In the same way, brains of the five 9L gliosarcoma-bearing rats that had undergone surgery alone and brains of the five rats that had undergone both surgery and gastrin release were also submitted to histopathologic examination (Fig. 5).

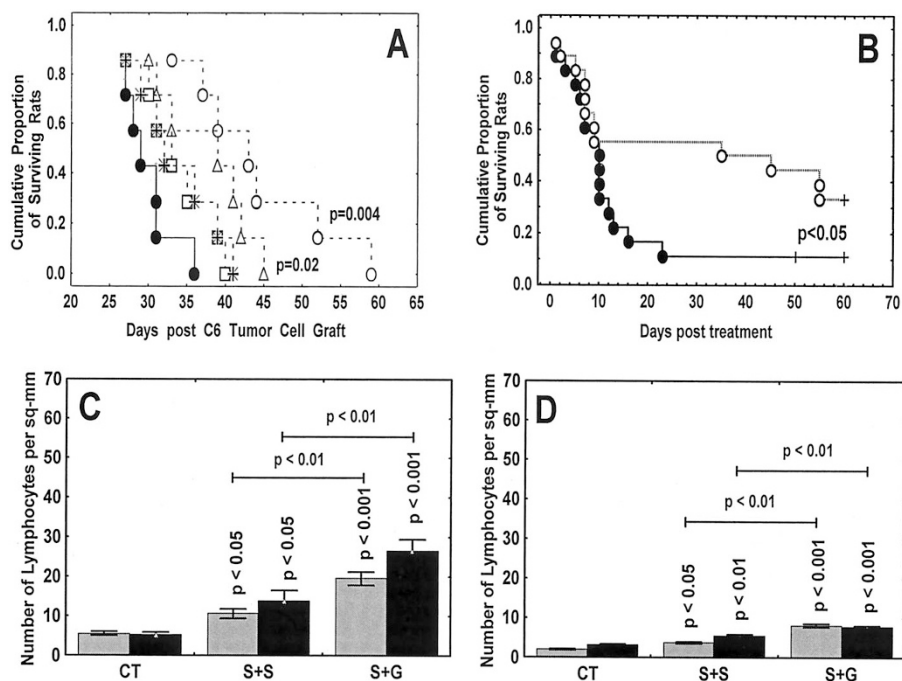


Figure 4.

A and B, Survival curves for the C6 glioma-bearing rats. A, the C6 cells were cultured for 24 hours in the presence of 10^{-12} M (asterisks), 10^{-10} M (open triangles), 10^{-8} M (open circles), and 10^{-6} M (open squares) gastrin or its absence (control: filled circles), before being stereotactically implanted (100,000 C6 cells/50 μ l/rat) into the rat brains. B, Fifty rats each had 100,000 C6 cells stereotactically grafted into their brains. Weekly monitoring of the weight of each rat showed that 36 of 50 C6 glioma-implanted rats began to lose weight between 19 and 24 days after C6 tumor cell implantation. Nuclear magnetic resonance (NMR) imaging was performed on each of the 36 rats and evidenced huge tumors in each of their brains. Thanks to pinpointing by NMR imaging, each rat underwent surgery to remove the bulk of its C6 glioma. Surgery was carried out when a rat had lost 10% of its weight relative to its weight the previous week. Micropumps were installed subcutaneously on the backs of the rats during surgery, immediately after the removal of C6 glioma bulk. The pumps delivered either saline (filled circles in B; 0.25 μ l per hour) or 10^{-8} M gastrin (open circles in B; 0.25 μ l per hour) for 14 days. Each group contained 18 rats. Two rats in Group A (without gastrin) and six rats in Group B (with gastrin) were long-term survivors; they were killed 60 days after surgery for the histopathologic examination of their brains (Fig. 5). C and D, Determination of the number of lymphocytes per square millimeter of brain tissue in three groups of rats implanted with C6 gliomas (C) or 9L gliosarcomas (D). Ten histologic fields were analyzed at magnification $\times 200$ for two tissue types for each brain analyzed, ie, the brain parenchyma surrounding the tumor (shaded bars) versus the tumor itself (black bars). The first group (CT group in C and D) included five rats implanted with C6 gliomas (C) or 9L gliosarcomas (D) and killed 14 days after the stereotactic implantation of 100,000 C6 or 40,000 9L tumor cells. The second group (S+S = surgery + saline) included the two long-term C6 survivors and five 9L tumors, whereas the third group (S+G = for surgery + gastrin) included the six long-term C6 survivors and five 9L tumors.

We used three groups of rats implanted with either C6 gliomas (Fig. 4C) or 9L gliosarcomas (Fig. 4D) to quantify the number of lymphocytes per square millimeter of brain tissue. For each brain analyzed, 10 histologic fields were analyzed at magnification $\times 200$ for two tissue types: first, the brain parenchyma surrounding the C6 glioma or the 9L gliosarcoma (shaded bars); and then the C6 glioma or the 9L gliosarcoma itself (black bars; Fig. 4, C and D). The first group included five rats implanted with C6 gliomas or 9L gliosarcomas and killed 14 days after the stereotactic implantation of 100,000 C6 or 40,000 9L tumor cells. The second group (S+S = surgery + saline) included the two long-term C6 survivors from Group A and five 9L tumors. The third group (S+G = surgery + gastrin) included the six long-term survivors from Group B and five 9L tumors. The data detailed in Figure 4, C and D show that lymphocytes were present in the brain parenchyma surrounding the C6 gliomas, in the gliomas themselves (Fig. 5), in the brain parenchyma surrounding the 9L gliosarcomas, and in the 9L gliosarcomas themselves. This was the case even with the rats that had not undergone treatment. Surgery without gastrin delivery significantly increased the

lymphocyte invasion in both the C6 gliomas and the 9L gliosarcomas. These features occurred both in the brain parenchyma surrounding the tumors and in the tumors themselves. Surgery and then gastrin delivery markedly increased the lymphocyte invasion as compared with what happened in the other two groups, the C6 gliomas and the 9L gliosarcomas. The effects were more pronounced in the allogenic C6 model than in the 9L syngenic model (Fig. 4, C and D).

It should be emphasized that lymphocyte counting was not performed at the same time after the delivery of gastrin to the C6 as in the 9L tumors. We performed this counting when the tumor-bearing animals had begun to die; the 9L tumor-bearing rats (treated by gastrin with surgery or left untreated) died sooner than the C6 tumor-bearing ones.

The low level of success attained by surgery alone in curing the animals bearing the C6 gliomas (2 of 18; Fig. 4B), or the 9L gliosarcomas (0 of 5; not shown), seems to relate to the highly invasive nature of these C6 and 9L models (Fig. 5). Figure 5A illustrates the highly invasive nature of the C6 glioma with respect to rat brains. Figure 5B illustrates the high proliferative activity of this C6 glioma. Figure 5C illustrates the morphology of a rat

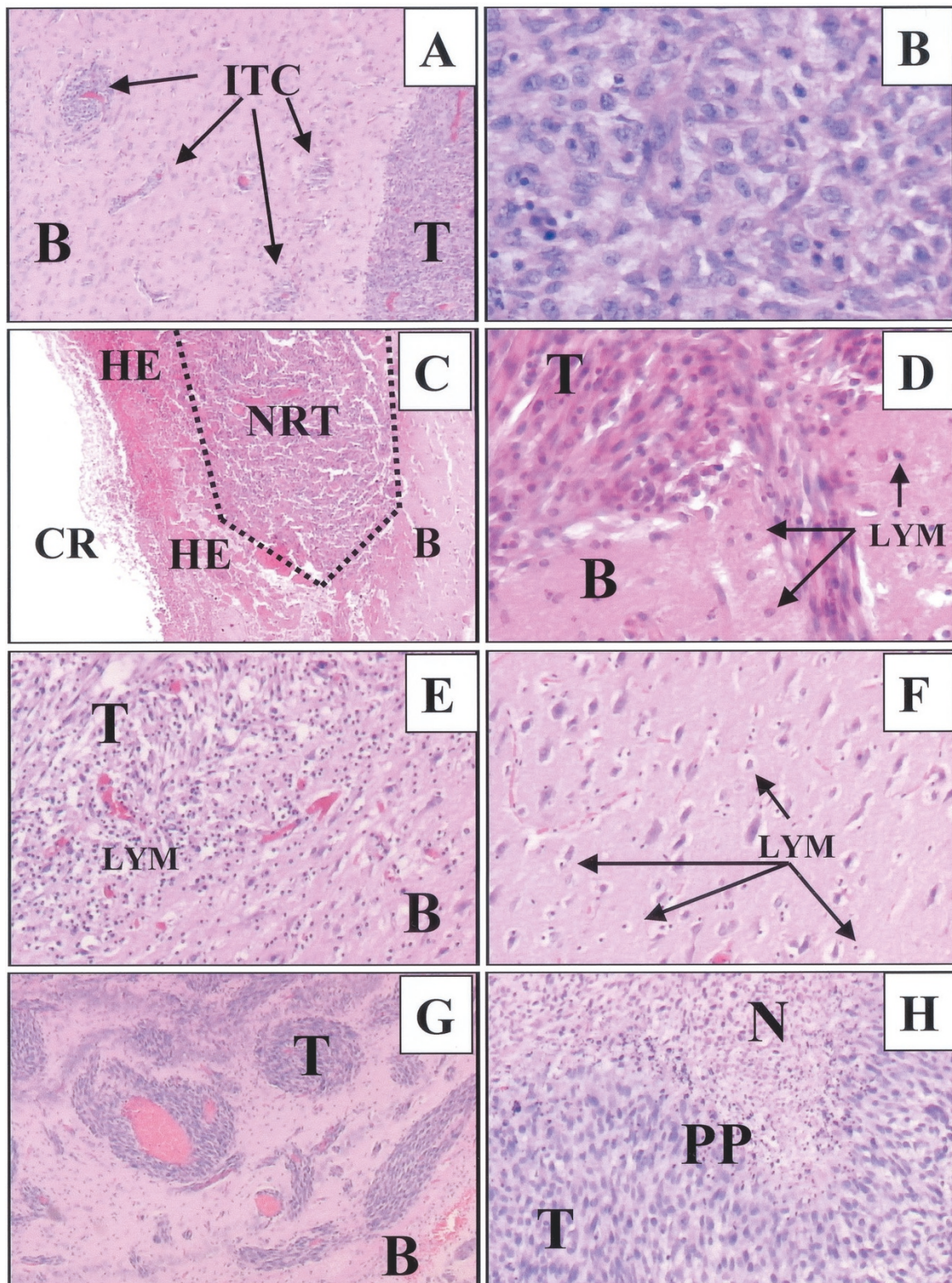


Figure 5.

Histopathologic examinations of the brains of the C6 glioma- and 9L gliosarcoma-bearing rats submitted to various experimental conditions. A, (original magnification, $\times 40$) and B, (original magnification, $\times 400$). The histologic appearance of a C6 glioma implanted into the brain of a rat not otherwise treated. B, presence of mitoses and lymphocytes. C, (original magnification, $\times 40$) and D, (original magnification, $\times 400$). The histologic appearance of the brain of a rat that had undergone surgery, with saline then delivered to the resection cavity for 1 week by means of the micropump. The rat died 12 days after surgery. E, (original magnification, $\times 100$) and F, (original magnification, $\times 100$). The delivery of gastrin into the cavity of resection in the brain of a C6 glioma-bearing rat resulted in a marked lymphocyte invasion of the surgical resection site and in the surrounding brain parenchyma. G, The 9L gliosarcoma exhibits dramatic invasive features. H, The 9L gliosarcoma exhibits pseudopalisading processes surrounding necrotic areas adjacent to the tumor bulk. B, brain; CR, cavity of resection; HE, hemorrhagic area; ITC, infiltrating tumor cell; LYM, lymphocytes; N, necrotic area; NRT, nonresected tumor; PP, pseudopalisading processes; T, tumor.

brain after surgery; saline was delivered to the resection cavity for 1 week by means of a micropump, and the rat died 12 days after surgery. Hemorrhagic areas and wound healing processes were present, as were unresected parts of the C6 glioma (Fig. 5C). Lymphocyte invasion was weak in this brain, the rat underwent surgery and saline (but not gastrin) delivery into the tumor resection cavity (Fig. 5C). Such lymphocyte invasion was also marked both in the tumor itself and in the brain parenchyma surrounding the tumor (Fig. 5D). Lymphocyte invasion was also weak in a C6 glioma implanted into the brain of a rat that did not undergo surgery (Fig. 5B). However, the presence of even a low percentage of lymphocytes emphasized the allogenic nature of the C6 gliomas grafted into the Wistar rats. The delivery of gastrin into the tumor resection cavity of a rat that underwent both surgery and gastrin delivery resulted in a marked lymphocyte invasion of the tissue surrounding the surgical resection cavity and, consequently, the gastrin delivery site (Fig. 5E). This invasion also affected the brain at a distance from the resection site (Fig. 5F).

The 9L gliosarcoma model exhibited very aggressive biologic characteristics (Fig. 5G), with some histopathologic features representative of high-grade gliomas including, for example, pseudopalisading processes surrounding large areas of necrosis in the tumor bulk (Fig. 5H).

Discussion

The present study deals with the characterization of gastrin-induced effects on glioma progression. Normal astrocytes possess significant amounts of CCK_B receptors (Hosli and Hosli, 1994), and this type of gastrin receptor might be a major primary target for CCK/gastrin peptides in the CNS (Muller et al, 1997). Tumor astrocytes deriving from low-grade astrocytic tumors (ie, WHO Grade II astrocytomas) also frequently exhibit significant amounts of CCK_B receptors (Reubi et al, 1997). In contrast, high-grade astrocytic tumors (WHO Grade IV glioblastomas) express no CCK_B receptors (Reubi et al, 1997). The present data suggest that CCK_B receptors could be replaced by CCK_C receptors in high-grade astrocytic tumors (Fig. 1). The present study shows that whereas U373 tumor astrocytes do not exhibit the mRNAs of CCK_A or CCK_B receptors, they do exhibit the mRNAs of CCK_C receptors (Fig. 1). Although C6 cells do not exhibit the mRNAs with respect to CCK_A receptors, mRNAs of CCK_B receptors are present (Fig. 1). The reverse feature was observed with respect to the 9L tumor cells. The C6, 9L, and U373 tumor cells produce weak, but nevertheless significant, amounts of gastrin in their culture media (Fig. 1).

We have already shown that gastrin triggers significant effects with respect to both the cell proliferation (Camby et al, 1996) and the migration (De Hauwer et al, 1998) features of tumor astrocytes. Using a subtractive hybridization PCR technique we cloned genes differentially over-expressed in U373 human astrocytoma cells treated with gastrin or left untreated (Ku-

charczak et al, 2001). We found approximately 70 genes over-expressed by gastrin, among which we identified the tenascin-C, the S100A6 (calcyclin), and the MLCK genes (Kucharczak et al, 2001), all of which are directly involved in cell migration features (Cramer and Mitchison, 1993; Lauffenburger and Horwitz, 1996). We also showed by means of a reporter gene system that the promoters of calcyclin and tenascin-C were up-regulated after gastrin treatment (Kucharczak et al, 2001). We also identified calcyclin as a prognostic marker in human astrocytic tumors (Camby et al, 1999, 2000).

Unlike U373 cells, C6 cells exhibit CCK_B gastrin receptors. In the present study, we chose the C6 rat and the U373 glioma models because they exhibit distinct types of gastrin receptors. The data that we obtained in previous *in vitro* studies all clearly indicated that gastrin targets multiple genes in tumor astrocytes, with marked consequences at proliferation and migration levels. However, no study to date has reported the effects mediated *in vivo* by gastrin on glioma progression. Even when in these earlier studies we identified some genes that seemed to be more or less specific targets for gastrin, we offered no explanation regarding to how gastrin is able to modulate cell proliferation in tumor astrocytes, nor how it modulates tumor astrocyte migration.

In the present study, we demonstrated both *in vitro* and *in vivo* that gastrin significantly modifies actin polymerization/depolymerization dynamics and, consequently, the organization of the actin cytoskeleton, and this could at least partly explain the gastrin-induced modifications to tumor astrocyte migration. These gastrin-induced modifications observed in the organization of the actin cytoskeleton are mediated, at least in part, by a gastrin-induced decrease in RhoA expression. RhoA, is known to be one of the most potent regulators controlling the organization of the actin cytoskeleton (Chrzanowska-Wodnicka and Burridge, 1996; Hall, 1998; Klemke et al, 1998). Taniguchi et al (1996) showed that gastrin targets RhoA, which, in turn, modifies the organization of the actin cytoskeleton. These authors also showed that CCK and gastrin promote the growth of fibroblastic NIH3T3 cells into which the CCK-B/gastrin receptor has been introduced, and that these peptides regulate stress fiber formation in an Rho/p21-dependent manner. They thus argue that the signals from CCK-B/gastrin receptors might affect cell growth, cell motility, and cell adhesion by regulating the actin cytoskeleton, a point that has recently been corroborated by Danen et al (2000), who show that RhoA is able to interact with several cyclin/cdk complexes in driving cells through the G1 phase of the cell cycle.

The gastrin-induced activation of various biologic processes is mediated by mitogen-activated protein kinase (MAPK)-induced *c-fos* gene transcription via protein kinase C (PKC)-dependent and C (PKC)-independent pathways (Stepan et al, 1999a, 1999b). Among other things, gastrin acts on the *Sis*-inducible element (SIE), the serum response element (SRE), and the Ca²⁺/cAMP response element (CRE), all of which

regulate the *c-fos* promoter (Stepan et al, 1999a, 1999b). Although activation of the SRE involves the small GTP-binding protein RhoA, gastrin targets the *c-fos* promoter CarG sequence via the RhoA-dependent pathways that play major roles in cell motility (Goodson et al, 1997; Maekawa et al, 1999). As mentioned above, gastrin can activate the PKC-dependent pathway that plays a dramatic role in tumor astrocyte migration (Janik et al, 1992; Zhang et al, 1997).

The data detailed in Figure 4, C and D show that lymphocytes are present both in the brain parenchyma surrounding C6 gliomas and in the gliomas themselves. This also applies, but to a lesser extent, to 9L gliosarcomas. This phenomenon could relate to the allogenic nature of the C6 glioma model as opposed to the syngeneic nature of the 9L gliosarcoma model (Parsa et al, 2000). In both models (Fig. 4, C and D), surgery without gastrin delivery significantly increased lymphocyte invasion compared with the control group. This feature also occurred both in the brain parenchyma surrounding the gliomas and in the gliomas themselves, and could relate to the endogenous release of gastrin and/or cholecystokinin by rats stressed by anesthesia and surgery (Bhatnagar et al, 2000; Koks et al, 2000; Tsutsumi et al, 1999). In stressed rats, gastrin/CCK peptides are probably secreted from neurons in the lateral parabrachial, the periaqueductal gray matter, and/or the dorsal raphe nuclei (Bhatnagar et al, 2000). Surgery and gastrin delivery markedly increased lymphocyte invasion compared with the other groups (Fig. 4, C and D). The phenotypical profiles could well be different in the case of the lymphocytes present in and around the C6 gliomas and the 9L gliosarcomas from the rats that underwent no further treatment, in the case of the lymphocytes in the rats that underwent surgery alone, and in the case of the lymphocytes in the rats that underwent surgery followed by gastrin delivery. We are now characterizing these immunologic profiles. In addition, whether these lymphocytes are activated against C6 and 9L tumor cells remains to be seen. It also remains to be seen whether gastrin induces specific immunomodulatory effects on the lymphocytes that it recruits, and particularly in addition to its chemotactic influence evidenced both here (Figs. 4 and 5) and earlier by de la Fuente et al (2000).

In conclusion, our data show clearly that *in vitro* gastrin is able to significantly modulate the invasion and motility features of C6 and U373 cells. Gastrin significantly increased the survival periods of the C6 glioma-bearing rats, not only through modifications in the context of tumor astrocyte migration features, but possibly also through immunomodulatory influences including lymphocyte recruitment in the C6 gliomas.

Materials and Methods

Cells and Compounds

The C6 rat glioma, the 9L rat gliosarcoma, and the U373 human glioma models were obtained from the

American Type Culture Collection (ATCC, Manassas, Virginia) and were maintained as monolayers. Also ordered from the ATCC were the human Jurkat (acute T cell leukemia) cells that we used as a positive control for CCK_B receptor determination, and the human LoVo colon cancer cells that we used as a positive control for CCK_C receptor determination. Gastrin (pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH₂) was purchased from Sigma (St. Louis, Missouri). The L-365,260 compound (3*R*-3-[*N'*-(3-methylphenyl)ureido]-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one) was obtained from ML Laboratories PLC (London, United Kingdom).

Analysis of CCK_A, CCK_B, CCK_C, and Gastrin RNA Expression

The U373, C6, and 9L tumor cells were grown to subconfluence in plastic Falcon dishes (Nunc-Gibco BRL, Life Technologies, Merelbeke, Belgium) and lysed by adding Tripure Isolation Reagent (Roche, Mannheim, Germany). Total RNA was prepared according to the manufacturer's recommendation. Before cDNA synthesis, the RNA was incubated with DNaseI (1.71 IU/ μ l) (Roche Diagnostics, Mannheim, Germany) for 15 minutes at 37° C. The RNA was purified by phenol/chloroform extraction. One microgram of total RNA was used as a template for cDNA synthesis. Reverse transcription was performed for 50 minutes at 42° C in RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 10 mM DTT; Gibco), oligodT (12–18) primers (25 ng/ μ l; Gibco), 500 nM dNTP, 8 IU RNasin Ribonuclease Inhibitor (Promega, Leiden, The Netherlands), and 200 IU Superscript RNase H Reverse Transcriptase (Gibco). The reaction was terminated by incubation for 15 minutes at 70° C.

Integrity of the cDNA Confirmed by β -actin Specific PCR Analysis

The primers for the CCK_A and CCK_B receptors and gastrin-17 were designed using the HYBSimulator software (Advanced Gene Computing Technologies, Irvine, California) (Hyndman et al, 1996), and were purchased from BioSource Europe S.A. (Nivelles, Belgium). The primers used for C6 and 9L rat tumor cells were CCK_A: 5'-CTTCATGAACAAACGCTTTCG-3' sense and 5'-CCATAATTCTACAGGAGCAGAA-3' antisense; CCK_B: 5'-ACAGATAGGAGTCTCATAGGA-3' sense and 5'-ACCTACCTCAAGAAGTCCA-3' antisense; and gastrin-17: 5'-GTGCATGCTAGTCTTAGTGC-3' sense and 5'-AGCATTGCTAGTTATACTGGTC-3' antisense.

PCR Products Analyzed by Electrophoresis on 1% Agarose Gels

Because the presence of a third CCK receptor, the 72-kd CCK_C receptor (Baldwin, 1994), has been reported in human tumors, we assayed the presence of CCK_A, CCK_B, and CCK_C mRNAs in the U373 human glioma model. RNA extraction, DNase treatment, and

Reverse Transcription were performed as described above for the C6 and 9L rat tumor cells.

The primers for the CCK_A, CCK_B, and CCK_C receptors and gastrin-17 were designed using the HYB-simulator software and were purchased from Bio-Source Europe S.A. (Nivelles, Belgium). The primers used were: CCK_A: 5'-CTGCTCAGCTGCTGGGAAAC-3' sense and 5'-CGGGACTGTAAGGGTTTGC AAAT-3' antisense; CCK_B: 5'-CCCATAGAAACATGACACTG -3' sense and 5'-GAGATTAGGCCTGATGGTATT-3' antisense; CCK_C: 5'-TGGATAGTATTTTAGCGAGTCTG-3' sense and 5'-CTTCTCGTTACTCTGATAAATCTAG-3' antisense; and gastrin-17: 5'-CGAGATGCAGCGACT-ATG-3' sense and 5'-GTTCTAGGATTGTTAGTTC-ATC-3' antisense.

Quantitative ELISA Determination for Gastrin Production in Tumor Cells

The level of gastrin-17 secreted by C6, 9L, and U373 tumor cells in their culture media was evaluated by means of a 96-well ELISA assay (Biohit Plc, Helsinki, Finland) following the manufacturer's instructions. Briefly, this assay is based on a sandwich enzyme immunoassay using horseradish peroxidase conjugated with a biotin/avidin amplification system. This immunoassay is specific for gastrin-17 and no cross-reactivity is observed with cholecystokinin, gastrin-34, or minigastrin.

Quantitative Western Blotting Analyses

Cytosol extracts from the C6 cells were prepared as described by Andrews and Faller (1991). Protein extracts containing 30 μ g (evaluated by the BCA protein assay; Pierce, Polylabo, Antwerp, Belgium) were loaded into each lane of a polyacrylamide gel (12%) under denaturing and reductive conditions. After electrophoresis, the proteins were transferred onto a Polyscreen PVDF membrane (NEN Life Science Products, Boston, Massachusetts) by tank blotting. RhoA proteins were further immunodetected by specific affinity-purified monoclonal mouse-anti-RhoA (1/1,000; Neomarkers) antibodies in conjunction with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (0.2 μ g/ml; NEN Life Science Products). The levels of expression of the RhoA proteins were quantitatively determined by means of a gel scanner and a Bioprofil Image Analysis Software (Vilber Lourmat, Marne La Vallée, France), as detailed previously (Belot et al, 2001; Nagy et al, 2001; Steinfeld et al, 2000).

Determination of In Vitro Cell Motility

The C6, 9L, and U373 cell motility levels were characterized by means of a device described elsewhere (De Hauwer et al, 1998; Delbrouck et al, 2002; Nagy et al, 2001) that enables the trajectories of living cells maintained in culture to be quantified. The greatest linear distance migrated by each cell was calculated from these trajectories. This distance refers to the maximum relative distance from the point of origin (the

MRDO quantitative variable) (De Hauwer et al, 1998; Delbrouck et al, 2002; Nagy et al, 2001). The experiments were performed over 24 hours, and one image was recorded every 4 minutes. Because each experiment was repeated independently three times, a minimum of 844 and a maximum of 1,008 cells were analyzed in each experimental condition.

Determination of In Vitro Cell Invasion

The C6, 9L, and U373 tumor cell invasion was determined by means of the thick collagen gel assay described by Vakaet et al (1991). The number (expressed as a percentage) of cells that migrated into a collagen matrix were recorded. Two independent sets of experiments were performed. The C6, 9L, and U373 tumor cells were cultured for 24 hours in the presence of four distinct doses of gastrin before being plated. Control took the form of C6 tumor cells cultured for 24 hours in the absence of gastrin before being plated onto the collagen matrix.

Quantitative Determination of Actin Amounts

The influence of gastrin concentrations of between 0.01 and 1,000 nM on the organization of the actin cytoskeleton was investigated by means of fluorescent probes, as we detailed elsewhere (Delbrouck et al, 2002; Nagy et al, 2001). Although fluorescent phalloidin conjugated with BODIPY-FL fluorochrome (Molecular Probes, Eugene, Oregon) was chosen to label the fibrillar actin, Texas red conjugated DNaseI (Molecular Probes) was chosen to label the glomerular actin. The relative quantification of filamentous and glomerular actin ratios was carried out by means of computer-assisted fluorescent microscopy based on the EXPLORER software (SambaTechnologies, Grenoble, France), as detailed elsewhere (Delbrouck et al, 2002; Nagy et al, 2001).

In Vivo Survival Analyses

C6 Tumor Cells Preincubated In Vitro with Gastrin. Five groups of 8 rats had 100,000 cells stereotactically implanted into their right frontoparietal lobes. The first group received C6 cells cultured in standard medium. The second, third, fourth, and fifth groups received C6 cells incubated before stereotactical implantation for 24 hours in media containing 10^{-12} , 10^{-10} , 10^{-8} and 10^{-6} M gastrin, respectively. The animals' survivals were recorded. This experiment was repeated 6 months later.

C6 and 9L Tumor Cells Stereotactically Implanted into Rat Brains and then Treated with Gastrin. Fifty rats had 100,000 C6 cells stereotactically grafted into their brains exactly as detailed above for the implantation of the gastrin-pretreated C6 cells. Weekly monitoring of the weight of each rat showed that 36 of 50 C6 glioma-implanted rats began to lose weight 19 to 24 days after C6 tumor cell implantation. NMR imaging performed on each of these 36 rats evidenced a huge brain tumor in each case. Thanks to the localization of the gliomas by NMR imaging, each of the rats under-

went surgery to remove the bulk of its C6 glioma. Surgery was carried out on each rat when it had lost 10% of its weight, compared with its weight measured the previous week.

During surgery, a micropump (Alzet micro-osmotic pump, model 1002; Alza Corporation, Palo Alto, California) was installed subcutaneously on the back of each rat, immediately after the removal of the C6 glioma bulk. These pumps delivered either saline (Group A; 0.25 μ l per hour) or 10^{-8} M gastrin (Group B; 0.25 μ l per hour) for 14 days. The A and B groups each contained 18 rats. Identical experimental procedures were carried out with the 9L model (see "Results"). All of the in vivo experiments described were performed with the authorization of the Animal Ethics Committee of the Faculty of Medicine of the Université Libre de Bruxelles (Agreement no. 55/LA 1230342).

Statistical Analyses

Statistical comparisons between the control and the treated groups were first made by the Kruskal-Wallis test (a nonparametric one-way ANOVA). In cases where this test revealed significant differences, we investigated whether any of the treated groups differed from the control. For this purpose, we applied the Dunn Procedure (two-sided test) for multiple comparisons adapted to the special case of comparisons between treatment and control, that is, only $k-1$ comparisons were made among the k groups tested by the Kruskal-Wallis test, instead of the possible $k(k-1)/2$ comparisons considered in the general procedure (Rosner, 1995; Siegel and Castellan, 1988). Survival analysis was performed by using Kaplan-Meier curves and Gehan's generalized Wilcoxon test. All statistical analyses were carried out using Statistica (Statsoft, Tulsa, Oklahoma).

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