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The endothelin system in Morris hepatoma-7777: an endothelin receptor antagonist inhibits growth *in vitro* and *in vivo*

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1 Plasma concentrations of endothelin are increased in patients with hepatocellular cancer as well as in patients with liver metastasis. However, the impact of these findings remains uncertain.

2 We thus analyzed the endothelin system in a rat hepatoma model (Morris hepatoma 7777) *in vitro* and *in vivo*.

3 Our study revealed that tissue concentrations of endothelin-1 (ET-1) and big-ET-1, the precursor of ET-1, were significantly elevated in Morris hepatoma 7777 as compared to normal liver. The ETA receptor density was significantly elevated, whereas the density of the ETB receptor was decreased in Morris hepatoma 7777.

4 We could also demonstrate that hepatoma cells secrete ET-1.

5 Exogenously added ET-1 enhances hepatoma cell growth in a dose-dependent manner. Endothelin receptor antagonists (ETA and combined ETA/ETB receptor antagonists) inhibit tumor cell growth *in vitro*. Since the combined ETA/ETB receptor antagonist was more effective *in vitro*, we used this compound also for *in vivo* studies and could demonstrate that a combined ETA/ETB receptor antagonist is able to reduce hepatoma growth *in vivo*.

6 In conclusion, the endothelin system is activated in Morris hepatoma 7777 and contributes to hepatoma growth. Since endothelin receptor antagonists are well-tolerated upcoming clinically used drugs without major side effects, our data might provide a new pharmacological approach to reduce hepatoma growth *in vivo*.

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Abbreviations: ET, endothelin; MH, Morris hepatoma; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide; VEGF, vascular endothelial growth factor

Introduction

Endothelins (ET-1, ET-2 and ET-3) are a family of 21 amino-acid peptides involved in the regulation of vasomotor tone. ET-1 was the first to be characterized in the supernatant of cultured vascular endothelial cells (Yanagisawa *et al*., 1988). The biological effects of endothelins are mediated by plasma membrane-bound receptors (ETA and ETB receptor). They belong to the family of rhodopsin-like receptors coupled to G-proteins. Apart from being a recognized vasoconstrictor peptide, ET-1 has been shown to be a growth-promoting peptide stimulating *in vitro* proliferation of nonmalignant cells such as fibroblasts, smooth muscle cells and mesangial cells. In addition, it was also shown that ET-1 promotes growth of several tumor cell lines including melanoma, prostate, colorectal and ovarian cancer cells (Battistini *et al*., 1993; Nelson *et al*., 1995; 1996; Kikuchi *et al*., 1996; Nelson *et al*., Moraitis *et al*., 1997; Ali *et al*., 2000a, b). Moreover, ET-1 tissue concentrations were found to be elevated in prostate and colorectal cancer (Kojima & Nihei, 1995; Nelson *et al*., 1995; Asham *et al*., 1998; Simpson *et al*., 2000; Asham *et al*., 2001). In line with these findings, studies are demonstrating that endothelin receptor antagonists can inhibit tumor cell growth in prostate and colorectal cancer and also in melanoma cells (Nelson *et al*., 1995; Lahav *et al*., 1999; Asham *et al*., 2001).

There are several studies indicating that plasma concentrations of ET-1 are elevated in human hepatocellular cancer (Ishibashi *et al*., 1993; Nakamuta *et al*., 1993). However, the main limitation of these important studies is the paracrine nature of this hormone system. Endothelins are locally acting tissue hormones. Thus, tissue concentrations of components of this system are more important. In the current study, we therefore analyzed tissue concentrations of ET-1 and big-ET-1 as well as the expression of both endothelin receptors (ETA and ETB) in a rat model of hepatocellular cancer, the Morris hepatoma. These tumors are transplantable liver tumors in rats. The primary tumor investigated in our study, Morris hepatoma 7777, was induced in a female buffalo rat by administration of FPA (*N*-2-fluorenylphthalamic acid)

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(Morris, 1965; Morris & Wagner, 1968; Hocher *et al*., 1994). Besides the characterization of the endothelin system in this hepatocellular cancer, we performed *in vitro* and *in vivo* studies in order to analyze the effects of endothelin receptor antagonists on tumor cell growth (*in vitro*) and hepatoma growth (*in vivo*).

Methods

Chemicals

[^{125}I]ET-1 (81400 TBq mol^{-1}) was obtained from Du Pont (Belgium). Unlabeled ET-1 was from Sigma (Germany). The endothelin receptor antagonists (LU 135252 and LU 302872) were a generous gift from Knoll (Germany). The selective endothelin receptor ligands BQ 123 and BQ 3020 were purchased from California Peptides (U.S.A.). The ET-1/big-ET-1 enzyme immunoassay was purchased from Biomedica (Austria). The colorimetric BrdU cell proliferation ELISA was purchased from Boehringer-Mannheim (Germany). Unless otherwise stated, all other reagents were of analytical grade and were purchased from Biochrom, Boehringer-Mannheim, Merck and Sigma (all Germany).

Cells

The MH-7777 cells were generously provided by Professor Dr R. Tauber (Charité Berlin, Germany).

Hepatomas

All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals. Buffalo rats were maintained on standard laboratory chow (Altromin, Germany) and tap water with a 12 h light–dark cycle at constant temperature and humidity.

Buffalo rats received bilateral transplantation of MH-7777 in the femoral musculature. Tumor mince (1 ml) was diluted with 0.5 ml 0.9% NaCl and 0.5 ml of the resulting suspension was injected using a 20-g needle. This tumor model is well established in our laboratory (Riese *et al*., 1987).

ET-1/big-ET-1 enzyme immunoassay

Plasma and tissue ET-1 and big-ET-1 concentrations were determined as recently described (Hocher *et al*., 1999). Briefly, the animals were killed 21 days after transplantation of hepatomas. Livers and hepatomas were frozen immediately in liquid nitrogen and stored at −70°C. Plasma samples were taken from the retro-orbital vein plexus and frozen at −20°C. The frozen tisssue (without necrotic parts of the tumor) was pulverized using a Micro-Dismembrator (B. Braun, Germany) and subsequently suspended in 2.8 ml per gram frozen weight of phosphate buffer (140 M NaCl, 2.6 M KCl, 8 M Na_2HPO_4, 1.4 M KH_2PO_4, 1% Triton-X). After homogenization followed ultracentrifugation at 4°C for 60 min at 100 000 × *g*. The supernatant and the plasma samples were used to perform the purchased ET-1/big-ET-1 enzyme immunoassay based on a purified polyclonal capture antibody and a monoclonal detection antibody. The (immunoreactive) ET-1 and (immunoreactive) big-ET-1 concentrations were related to protein concentrations.

Endothelin receptor-binding experiments

Membranes were prepared according to Hocher *et al*. (1998). Approximately 0.4 g of liver or hepatoma was homgenized at 4°C in 10 ml of 20 M $NaHCO_3$ using a motor-driven pestle homogenizer. The homogenate was centrifuged at 4°C for 15 min at 1000 × *g*. The supernatant was decanted and centrifuged at 4°C for 30 min at 40,000 × *g*. The pellet consisting of crude plasma membranes was resuspended in 1.5 ml assay buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4).

In order to analyze the expression of endothelin receptor subtypes (endothelin ETA receptor, endothelin ETB receptor) in liver and hepatoma, binding assays were performed in the presence or absence of the subtype-specific endothelin receptor ligands BQ 123 (3 μm) or BQ 3020 (3 μm) (Hocher *et al*., 1995). The assay buffer contained 100 mM Tris-HCl, 5 mM $MgCl_2$, 1 g l^{-1} bacitracin and 1 g l^{-1} bovine serum albumin, pH 7.4, in a total volume of 150 μl. The [^{125}I]ET-1 tracer concentration was kept constant at 40,000 cpm per tube, while the concentration of unlabeled ET-1 was increased from 0 to 15 nM (competition studies with 'cold saturation'). Samples from crude plasma membranes were used at a protein concentration of 0.53 g l^{-1}. Binding studies were performed at room temperature for 120 min. Nonspecific binding was assessed in the presence of excess ET-1 (3.3 μM). After adding 1 ml of cold binding buffer, free and receptor-bound radioactivity was separated by centrifugation at 4°C for 15 min at 30,000 × *g* and the pellets thus obtained were washed two more times with 1 ml of cold binding buffer. [^{125}I] was counted in a Kontron Gamma Counter (78% counting efficiency for [^{125}I]). To exclude that endogenously bound ET-1 might influence the binding studies, we used in some control experiments the acid-wash technique to remove potentially bound endogenous ET-1 from the endothelin receptor prior to the binding studies. This technique was performed as described by Ullian & Linas (1989); Ullian & Linas (1990) and Hocher *et al*. (1992), with minor modifications.

Cell culture experiments

MH-7777 cells (Hocher *et al*., 1994) were cultured with DMEM (Boehringer Ingelheim Bioproducts, Belgium), containing 10% fetal calf serum, 10^5 U l^{-1} penicillin, 100 mg l^{-1} streptomycin, 1 mg l^{-1} human insulin and 0.4 mg l^{-1} dexamethasone in a humidified incubator with 5% CO_2 at 37°C. Cells were cultured in 96-well ELISA plates (Falcon, U.S.A.). About 6000 cells were seeded into each well. After 24 h, the medium was exchanged for fetal calf serum-free medium with different concentrations of ET-1, endothelin receptor antagonists (ETA receptor antagonist: LU 135252; combined ETA/ETB receptor antagonist: LU 302872; Raschak *et al*., 1995; Hocher *et al*., 2003) or vehicle alone. After 24 h, proliferation was assessed using the MTT assay (Mosmann, 1983). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was added to a final concentration of 2.1 g l^{-1} to each well. Cells were then incubated at 37°C for 4 h. SDS ($C_{12}H_{25}NaO_4S$) was added to a final concentration of 45 g l^{-1}. Cell cultures were left overnight until the blue product dissolved in the medium. Absorption was then measured at 570 nm.

In order to assess possible toxic effects of the receptor antagonists used, lactate dehydrogenase activity was measured in the supernatant of treated and untreated cells as well as in mechanically (aspiration through a thin needle) and chemically harmed cells (incubation with dimethylsulfoxide or nutrient-free medium).

Endothelin combined receptor *antagonist (LU 302872) treatment experiment* in vivo

MH-7777 cell suspensions were injected into both hindlimbs of 44 male Buffalo rats (Riese *et al*., 1987). The rats were randomly divided into two groups with 22 rats. The first group received placebo, the second group was treated with the combined ETA/ETB receptor antagonist LU 302872 added to the drinking water (mean 56.5 mg kg^{-1} day^{-1}). According to producers instructions, we prepared a 3.15 mmol l^{-1} solution of LU 302872 in tap water. The molecular weight of LU 302872 is 528.61 g mol^{-1}. LU 302872 (1.66 g) was disssolved in 31.4 ml 0.1 N NaOH. Tap water was then added to 1000 ml and the pH was adjusted to 7.5 using 0.1 N HCl. We calculated a fluid intake of 30–35 ml kg^{-1} day^{-1} to reach a dose of about 50–60 mg kg^{-1} day^{-1}. (The mean weight of the rats was 0.476 kg.) Similar doses are reported to be effective and free of major side effects in recent renal and cardiovascular studies (Wolf *et al*., 2002; Hocher *et al*., 2003). Treatment was started 12 h after MH-7777 cell injection into the hindlimbs. Rats were treated for 28 days. Fluid intake and bodyweight was measured every 5 days. For 28 days, the tumor growth was followed up by regular transcutaneous measurement using a precision caliper. The measurement was performed under anesthesia. This method was originally developed by Erdstein *et al*. (1984) and has been used for Morris hepatomas. Volume determinations using ultrasound were additionally performed (7.5 MHz, Kontron, Germany). The diameter of the hepatomas was determined in two planes. Assuming a rotation-elipsoid, the tumor volume was calculated as $4/3\pi ab^2$. At the time of killing, hepatomas and organs were excised, weighed and measured. The tumor volume was calculated as $4/3\pi abc$ as described by Linder-Horowitz *et al*. (1969), asssuming a spheroid. Blood samples were taken at study end and analyzed as recently described (Hocher *et al*., 2003).

Histology

The hepatomas were fixed, cut and a standard hematoxilin–eosin staining was performed. Evaluation of tumor morphology (necrotic area, cell hypertrophy and anaplasia of the nucleus, number of mitosis and apoptosis) was carried out by two investigators without knowledge of the treatment groups of each section.

Statistical analysis

Unless otherwise stated all results are expressed as mean ± standard error (s.e.). Statistically significant differences ($P<0.05$) among individual groups were determined using one-way ANOVA followed by unpaired *t*-test.

Results

ET-1/big-ET-1 plasma and tissue concentrations in Buffalo rats with and without MH-7777

The tissue ET-1 and big-ET-1 concentrations in MH-7777-tissue (21 days after transplantation) are significantly elevated compared to liver tissue of hepatoma-bearing and non-hepatoma-bearing animals (93.7% elevation for ET-1 and 37% for big-ET-1 compared to liver of non-hepatoma-bearing rats, see Figure 1).

Plasma concentrations, however, of ET-1 and big-ET-1 in hepatoma-bearing rats (ET-1: 24.3 ± 6.1 pmol l^{-1}; big-ET-1: 16.5 ± 2.2 pmol l^{-1}) and non-hepatoma-bearing rats (ET-1: 24.0 ± 3.9 pmol l^{-1}; big-ET-1: 15.7 ± 2.3 pmol l^{-1}) were not statistically different (*n* was 11 in each group).

ETA and ETB receptors in MH-7777 and liver tissue

The ETA receptor density is significantly elevated in hepatoma tissue compared to normal liver tissue. The ETB receptor density – on the other hand – is significantly decreased in hepatoma compared to normal liver tissue (Figure 2).

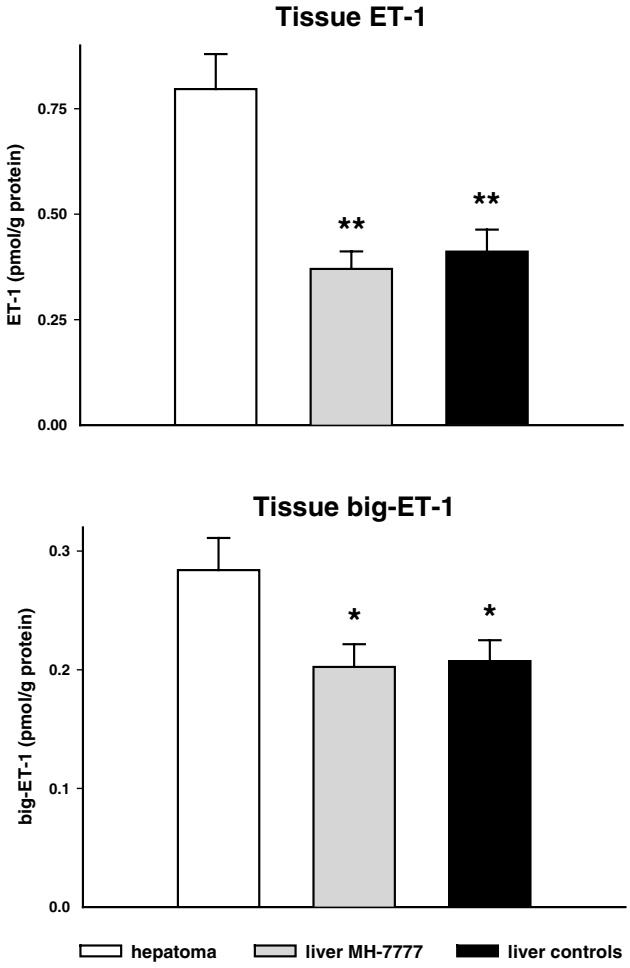

Figure 1 Tissue concentrations of ET-1 and big-ET-1 in hepatoma, liver of hepatoma-bearing and liver of non-hepatoma-bearing buffalo rats. $n=11$ per group. Data are given as mean ± s.d. $^*P<0.05$, $^{**}P<0.001$ vs hepatoma.

The Scatchard analysis revealed only one type of ETA and ETB receptor-binding sites in hepatomas and normal rat liver tissue. The ETA receptor affinity (K_d) was significantly higher in hepatomas compared to normal liver, whereas the ETB receptor affinity was similar in all groups (Table 1).

Potential relevant endogenous binding of ET-1 to its receptors that might influence the binding studies could be excluded by the acid-wash technique.

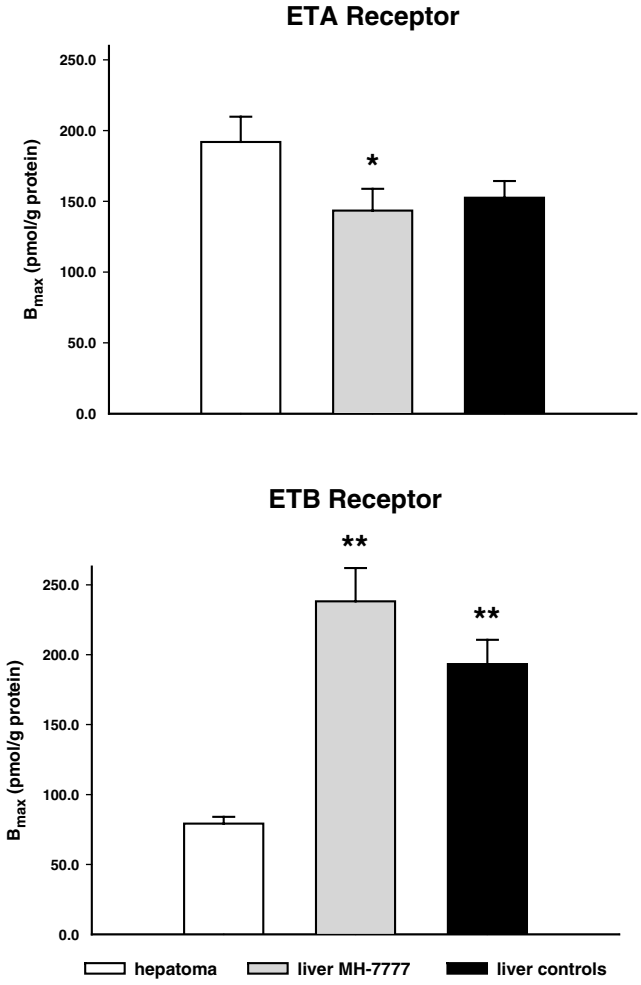

Figure 2 ETA and ETB receptor density in hepatoma, liver of hepatoma-bearing and liver of non-hepatoma-bearing buffalo rats. $n=11$ per group. Data are given as mean$\pm$s.d. $^*P<0.05$, $^{**}P<0.0001$ vs hepatoma (same receptor subtype). The ETA receptor density between hepatoma and control liver was statistically not significantly different ($P=0.08$).

Table 1 Binding affinity of ETA and ETB receptors in Morris hepatoma (MH)-7777 tissue, liver of hepatoma-bearing (liver MH) and liver of non-hepatoma-bearing (liver controls) Buffalo rats

K_d (pmoll^{-1})	*MH-7777*	*Liver MH*	*Liver controls*
ETA	757$\pm$62	992$\pm$121	1093$\pm$128*
ETB	470$\pm$25	444$\pm$41	533$\pm$49

Data were given as mean$\pm$s.d.; $n=11$ per group, $^*P<0.05$ vs ETA receptor binding affinity in hepatoma.

Growth of MH-7777 cells after incubation with ET-1 and endothelin receptor antagonists in vitro

We could demonstrate an endogenous production of ET-1 in the supernatant of hepatoma cells. By counting the cells and measuring ET-1 in the culture medium every 12 h, we could detect a mean ET-1 synthesis rate of the hepatoma cells of 10.3 ($\pm$1.7) fmol per 10^6 cells per 24 h.

Exogenously added ET-1 increases hepatoma cell proliferation in a dose-dependent manner. The maximal effect was seen at concentrations of 10^{-9} moll^{-1} ET-1 in the culture medium. A further increase of ET-1 in the culture medium does not further enhance proliferation (Figure 3).

Addition of an ETA receptor antagonist (LU 135252) to the culture medium significantly ($P<0.05$) decreases tumor cell growth by 50% at a concentration of this compound in the medium of 1×10^{-4} moll^{-1} or higher. A combined ETB/ETB receptor (LU 302872) antagonist was more effective. The 50% growth inhibition was seen at LU 302872 concentrations of 8×10^{-5} moll^{-1} LU 302872 or higher (Figure 4).

Both endothelin receptor antagonists showed no cell toxicity even at the highest concentrations of these compounds used in this study. The activity of lactate dehydrogenase (LDH) in the supernatant was not significantly elevated after incubation with the highest used concentrations of the receptor antagonists (113 and 122% of controls after incubation with LU 135252 and LU 302872). There was a marked increase of LDH activity after incubation with nutrient-free medium, dimethylsulfoxide and after mechanic cell destruction (178, 340 and 1962% of controls) indicating that LDH activity is a sensitive marker for cell damage/cell toxicity.

Growth inhibition of MH-7777 in vivo

The necessary size to measure the hepatomas transverse diameter transcutaneously using a precision caliper is reached 10 days after tumor cell suspension injection. At that point, there is no relevant difference between the groups. Three animals of each group died after anesthesia for measuring tumor diameter at day 10 (one rat per group) and day 20 (two rats per group). The following measurements show a slower

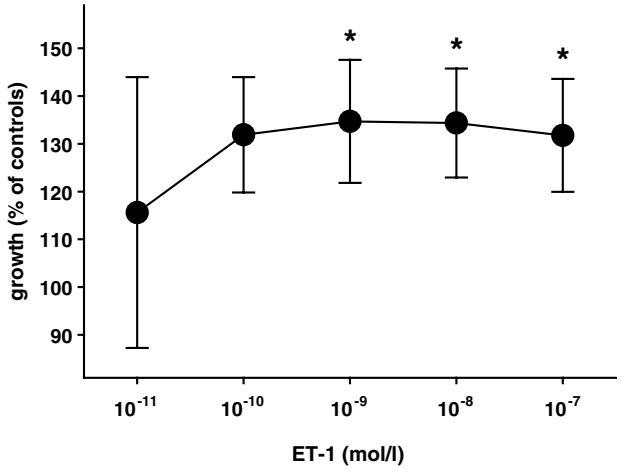

Figure 3 Cell growth of Morris hepatoma (MH)-7777 cells after 24 h incubation with ET-1; n was 5 per group for each point. $^*P<0.05$ vs controls (100%).

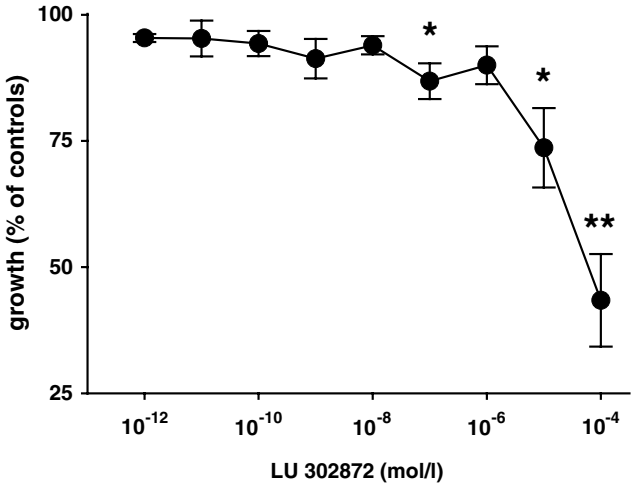

Figure 4 Cell growth of Morris hepatoma (MH)-7777 cells after 24 h incubation with the combined ETA/ETB antagonist LU 302872. $n=9$ per group for each point. $^{*}P<0.05$, $^{**}P<0.001$ vs controls (100%).

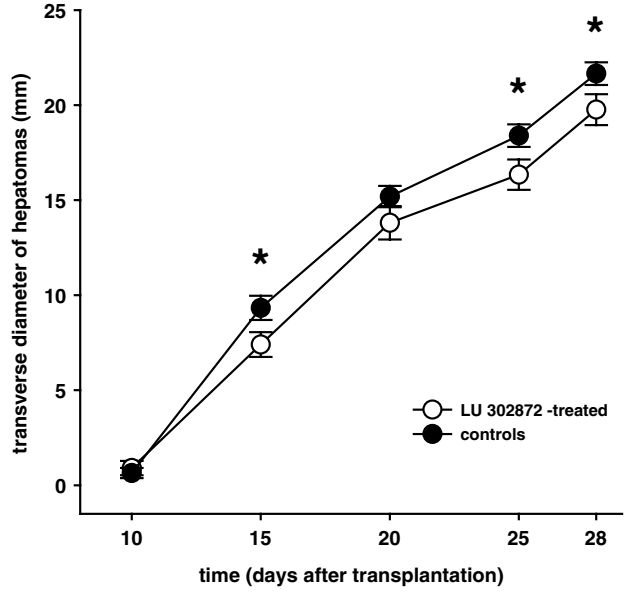

Figure 5 Transverse diameters of Morris hepatoma 7777 (MH-7777) in the hindlimbs of Buffalo rats. The diameters were measured using a precision caliper. LU 302872-treated vs untreated controls. $n=38$ (19 animals with two hepatomas in each group). $^{*}P<0.05$ vs controls at the same time.

tumor growth in the LU 302872-treated group. The mean tumor diameter in the treated group is only 79–91% of the mean diameter of the untreated group (days 15–28). The *in vivo* growth curves are significantly different (Figure 5) indicating that the combined ETA/ETB receptor antagonist LU 302872 significantly decreases tumor growth also *in vivo*.

Histological analysis of hepatomas

The histological evaluation revealed that there were no obvious differences between the two groups to be found in the hematoxilin–eosin staining. The hepatomas consist of mixed vital and necrotic parts. Hypertrophy and anaplasia of the nucleus, mitosis and apoptosis can be found in both groups. Interestingly, numerous perivascular necrosis can be seen in both groups. The number and aspect of blood vessels within and around the hepatomas showed no difference between the treated and nontreated group. Neither were there detectable differences in the healthy tissue surrounding the hepatomas in treated and nontreated rats bearing the Morris hepatoma.

Discussion

The present study revealed that Morris 7777 hepatoma cells secrete ET-1 *in vitro* and that ET-1 also promotes tumor cell growth. A combined ETA/ETB receptor antagonist – on the other hand – was able to inhibit Morris 7777 hepatoma cell growth *in vitro*. Furthermore, we analyzed this tumor *in vivo* and could demonstrate that tissue concentrations of big-ET-1, the precursor of ET-1, as well as tissue ET-1 concentrations are elevated in Morris hepatomas as compared to the healthy liver. The density of the ETA receptor was increased, whereas the density of the ETB receptor decreased markedly in Morris 7777 hepatomas as compared to the normal liver. In good agreement with our *in vitro* data was the finding that tumor growth of the Morris 7777 hepatoma could also be narrowed *in vivo* using a combined ETA/ETB receptor antagonist.

Plasma concentrations of big-ET-1 and ET-1 were not elevated in rats bearing Morris 7777 hepatomas. This is not a contradiction to the finding of elevated tissue concentrations of big-ET-1 and ET-1 in these animals. This rather reflects the paracrine nature of this hormone system (Hocher *et al*., 1997a). ET-1 transgenic mice with a high expression rate of the transgen in the kidneys and lungs are characterized by elevated kidney and lung tissue ET-1 concentrations, whereas plasma concentrations remain normal (Hocher *et al*., 1997b; 2000). Elevated plasma concentrations are generally seen, if vascular endothelial cells itself are damaged in conditions such as malignant hypertension or pregnancy-induced hypertension (Hocher *et al*., 1997a; Slowinski *et al*., 2002) and in conditions of pathological circulatory status such as heart failure and liver cirrhosis (Hocher *et al*., 1997a). However, there are some reports showing elevated plasma concentrations of ET-1 in humans with hepatoma (Ishibashi *et al*., 1993; Nakamuta *et al*., 1993; Uchida & Watanabe, 1993). This might be due to a spillover from hepatoma leading to increased plasma ET-1 concentrations or probably due to the comorbidity of the patients analyzed in these studies. Liver cirrhosis, for instance, is often associated with hepatocellular cancer and is on its own associated with an activation of the endothelin system (see above).

The expression pattern of endothelin receptors in Morris hepatoma 7777 (increased density of ETA receptors combined with a markedly decreased density of ETB receptors) seen in our study is obviously a typical pattern of the paracrine endothelin system in neoplasms. Nelson *et al*. (1995) were the first group describing this pattern in prostate cancer. While there is a predominance of ETB binding on human benign prostatic tissue, there is a strongly decreased ETB receptor expression in prostate cancer (Nelson *et al*., 1996). Similar

findings were reported in colorectal and ovarian cancer: an increased ETA and decreased ETB receptor density (Ali *et al*., 2000a, b; Bagnato *et al*., 1999). These findings suggest a general impact/role of the endothelin receptor pattern for tumor growth. Different mechanisms are discussed. In prostate, colorectal and ovarian cancer, the mitogenic effect of ET-1 is reported to be ETA mediated, the receptor subtype which is increased in these tumors as well as in MH-7777 (Nelson *et al*., 1996; Bagnato *et al*., 1999; Ali *et al*., 2000a, b; Asham *et al*., 2001).

Fukuroda *et al*. (1994) investigated which endothelin receptor subtypes (ETA and ETB) participate in ET-1 clearance from the blood. They found that especially the pulmonary ETB receptor is an important clearance receptor for circulating ET-1 in healthy rats. A decreased ETB receptor density would thus cause a decreased ET-1 clearance. However, it is so far unknown whether the ETB receptor in tumors might also act as a clearance receptor. Another study demonstrated that ET-1 inhibits proliferation of hepatic Ito cells *via* the ETB receptor (Mallat *et al*., 1995). A decreased ETB receptor density might therefore contribute to an enhanced cell proliferation. But once again, this pathway was not yet demonstrated in hepatoma cell lines. With respect to the ETB receptor, it is also important to note that ET-1 acts as an apoptosis survival factor for cultured rat endothelial cells. ET-1 dose-dependently suppressed the apoptosis induced by serum starvation. The ETB receptor antagonist BQ 788, but not the ETA receptor antagonist BQ123, blocked the apoptosis protective effect of ET-1. These data suggest that ET-1, as well as mitogen, functions as an apoptosis survival factor for endothelial cells in an autocrine/paracrine manner *via* the ETB receptor (Shichiri *et al*., 1997; 1998; Wu-Wong *et al*., 1997). A decreased ETB receptor density might thus cause less apoptosis of endothelial cells. This might promote tumor growth as well. However, all these potential mechanisms have to be analyzed in further studies addressing their impact on hepatoma cell growth.

The altered paracrine endothelin system in rats bearing Morris hepatoma 7777 is not only an epiphenomenon of malignant transformation of normal liver to hepatoma. By contrast, the endothelin system contributes to growth regulation of this experimental liver tumor. This notion is supported by the following findings:

(i) Exogenously added ET-1 promotes tumor cell growth *in vitro*.
(ii) Endothelin receptor antagonists reduce tumor cell growth *in vitro*.
(iii) Tumor growth of Morris hepatoma 7777 could also be reduced *in vivo* by an endothelin receptor antagonist.

The observed growth inhibiting effects of endothelin antagonists *in vivo* and *in vitro* are clearly not related to toxic side effects of these drugs (see the Results section and Table 2). Cell culture experiments revealed that a combined blockade of both endothelin receptors seems to be superior to a sole blockade of the ETA receptor. We thus performed the *in vivo* studies with a combined ETA/ETB receptor antagonist.

There are at least four potential mechanisms that might explain the antiproliferative effects of endothelin receptor antagonists *in vivo*. In colorectal cancer, endothelin-binding sites have been found mainly in tumor vessels and stroma tissue (Inagaki *et al*., 1992). There is evidence that endothelins may also have a role in vascularization of tumors. Pedram *et al*. (1997) demonstrated that both ET-1 and ET-3 comparably stimulated vascular endothelial growth factors (VEGF) production by vascular smooth muscle cells *in vitro* in a manner equipotent to hypoxia. The latter is an important known stimulus of VEGF production. This enhanced VEGF production led to increased invasion by endothelial cells of the extracellular matrix *in vitro*. Migratory effects on endothelial cells could be blocked by an ETB receptor antagonist (Ziche *et al*., 1995). It was also shown that ET-1 acts directly as a co-mitogen with other factors such as epidermal growth factor (Battistini *et al*., 1993; Bagnato *et al*., 1997). In a recent study, it was shown that an activation of endothelin receptors causes a transactivation of epidermal growth factor receptors leading to an enhanced mitogenic signaling in human ovarian carcinoma cells (Vacca *et al*., 2000). Blocking endothelin receptors thus also reduces the activity of growth factor receptors and might contribute to the reduced tumor cell growth after blocking endothelin receptors. Another recent study (Sauer *et al*., 2000) demonstrates that a G- protein-coupled signal transduction pathway that decreases intracellular cyclic AMP is involved in the regulation of Morris hepatoma cell growth. It was also shown that a mixed ETA/ETB receptor antagonist (like LU 302872) potentiates FasL-induced apoptosis in cultured colorectal cancer cells (Eberl *et al*., 2000).

Which of the mentioned mechanisms contributes to tumor growth in Morris hepatoma 7777 remains to be clarified in further studies.

Table 2 Concentrations of serum parameters describing liver and kidney function in treated and nontreated Morris hepatoma (MH)-7777-bearing rats ($n=16$ in each group) and controls without MH-7777 ($n=9$)

	Unit	*LU 302872*	*Untreated*	*Controls*
Albumin	$g\,l^{-1}$	32.3±0.4	32.7±0.4	32.7±0.4
Creatinine	$\mu mol\,l^{-1}$	19.6±0.6	19.3±0.4	16.2±0.5*
Cholesterol	$mmol\,l^{-1}$	2.8±0.11**	3.6±0.11**	2.3±0.10
Triglycerides	$mmol\,l^{-1}$	1.13±0.10	1.09±0.10	1.16±0.10
AST	$IU\,l^{-1}$	410±64	335±37	52±4*
ALT	$IU\,l^{-1}$	618±91	483±35	64±4*
LDH	$IU\,l^{-1}$	166±21	125±9	104±23
Glucose	$mmol\,l^{-1}$	9.9±0.6	11.4±1.1	8.1±0.4

Data are given as mean±s.d.; *$P<0.05$ vs both other groups. AST = aspartate aminotransferase, ALT = alanine aminotransferase, LDH = lactate dehydrogenase. *$P<0.05$ compared to treated and nontreated hepatoma-bearing rats; **$P<0.05$ compared to healthy control rats.

In conclusion, our study revealed that the endothelin system in Morris hepatoma 7777 is altered in a way that was also seen in other malignant tumors (increased tissue concentrations of ET-1 combined with an increased expression of ETA receptors and a markedly decreased density of ETB receptors). The activated endothelin system in Morris hepatoma is furthermore involved in tumor growth control. Since endothelin receptor antagonists are upcoming new drugs currently tested in humans in cardiovascular medicine without significant side effects, our study suggests that endothelin receptor antagonists offer a unique new therapeutic approach – most probably on top of established principles – to treat hepatocelluar cancer.

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