Response to Endothelin-1 in Arteries from Human Colorectal Tumours: Role of Endothelin Receptors

EDUARDO FERRERO,* MARIA LABALDE,* NURIA FERNÁNDEZ,† LUIS MONGE,† ADELY SALCEDO,† RAUL NARVAEZ-SANCHEZ,† MANUEL HIDALGO,* GODOFREDO DIEGUEZ,† AND ANGEL LUIS GARCÍA-VILLALON†,[1]

Departamento de Cirugía General y Digestiva (Section B), Hospital Universitario "12 de Octubre", Universidad Complutense, Madrid, Spain; and †Departamento de Fisiología, Facultad de Medicina, Universidad Autonoma, Madrid, Spain

To examine the reaction of tumour arteries to endothelin-1, we obtained arteries supplying blood flow to colorectal tumours from patients, as well as mesenteric arteries supplying the normal colon tissue from the same patients and mesenteric arteries from patients without a colorectal tumour pathology. The contraction in response to endothelin-1 and the relaxation produced by bradykinin was recorded in each of these arteries. Accordingly, the sensitivity to endothelin-1 but not the maximal response, was higher in the arteries supplying colorectal tumours than in mesenteric arteries supplying normal colon or in mesenteric arteries from patients with no tumour pathology. The contraction produced by endothelin-1 was not modified by exposure to L-NAME or meclofenamate in arteries supplying both the tumour and the normal colon. The endothelin ET_A and ET_B receptors were expressed similarly in arteries supplying the tumour or normal colon. However, the antagonist of the endothelin ET_B receptors BQ788 (10^{-6} M) decreased the contractions in the arteries supplying the tumour but not in those supplying the normal colon. By contrast, the antagonist of endothelin ET_A receptors BQ123 (10^{-6} M) reduced the contraction equally in both these types of arteries. Likewise, in arteries precontracted with U46619, the relaxation in response to bradykinin was similar in all three types of arteries. Together, these results suggest that the arteries supplying human colorectal tumours are more sensitive to endothelin-1, which could be due to the enhanced activity of endothelin ET_B receptors in the absence of any change in the modulatory effect of nitric oxide or prostanoids in the arterial response to this peptide. Exp Biol Med 233:1602–1607, 2008

Key words: endothelin ET_B receptors; nitric oxide; prostaglandins

Introduction

The tumour vasculature is a potential target for anticancer therapies that attempt to prevent tumour growth by impeding angiogenesis, thereby depriving the tumour of its blood and nutrient supply. However, strategies to increase tumour blood flow have also been proposed that may improve the delivery of chemotherapy agents to the tumour. Accordingly, a better understanding of how tumour blood vessels respond to vasoactive agents may be of particular relevance. There is evidence that tumour blood vessels may have morphological and functional features different to those of normal vessels. Indeed, arteries supplying different types of tumours, including those associated with colorectal tumours, display a progressive reduction in smooth muscle in the proximity of the tumour (1), as well as in arteries quite distant from the tumour (2). With regards to their functional characteristics, the increase in arterial blood pressure in rats is accompanied by an increment in the blood flow to transplanted or chemically induced tumours, without producing any changes in the blood flow to normal tissues, suggesting impaired autoregulation in the tumour vasculature (3). Intracarotid injection of histamine in rats with gliomas transplanted into the brain increased the blood flow to the tumour and surrounding tissue, but not in the contralateral brain hemisphere (4). Moreover rat aortas incubated with tumour cells from hepatocarcinoma or melanoma, display weaker contractions in response to phenylephrine and potassium chloride (5)

The vascular endothelium participates in the regulation of blood flow by releasing vasoactive factors that may

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[1] To whom correspondence should be addressed at Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 2, Madrid, Spain. E-mail: angeluis.villalon@uam.es

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produce relaxation, such as nitric oxide, or that may be vasoconstrictory like the endothelin-1 peptide. Endothelial function may be altered in tumours as there is reduction of nitric oxide release from blood vessels associated with experimental tumours in rats (6). Moreover, plasma endothelin-1 levels increase in patients with colorectal cancer (7), and the arterial contraction in response to endothelin-1 is reduced in rat fibrosarcoma (8) or increased in mouse liver tumour (9).

Studies on the response of tumour blood vessels to endothelin-1 have been performed in animals with experimental tumours, although to our knowledge, there are no studies of the response to this peptide in arteries from human tumours. Therefore, we decided to analyze the hypothesis that altered vascular reactivity may develop in the local tumour environment by assessing the effects of endothelin-1 on arteries from colorectal tumours, and comparing these effects with those in arteries supplying the normal colon in the same patients or from patients with no tumour pathology. As nitric oxide and/or prostanoids may modulate vasoconstriction in response to endothelin-1 (10), the influence of these factors on the response of mesenteric arteries supplying tumour or control tissue was studied using the inhibitors L-NAME and meclofenamate. The involvement of the ET_A and ET_B endothelin receptors may also be altered in pathological conditions (11) and thus, the response to endothelin-1 was studied in the presence of the specific ET_A and ET_B receptor antagonists, BQ123 and BQ788, as was the expression of these receptors in western blots. Together, the data obtained contribute to our understanding of the role of endothelin-1 in regulating the blood supply to tumour tissue, as well as the reactivity of tumour vasculature.

Methods

In this study, arteries (0.7–1.5 mm in external diameter) from 13 patients diagnosed with colorectal tumours were used (mean age 68 $\pm$ 3 years, 5 males and 7 females, 6 with arterial hypertension and one with arterial hypertension and non insulin-dependent diabetes mellitus). The study was approved by the local Ethical Committee and the informed consent from all the patients was obtained before they were allowed to participate. Arteries supplying blood flow to the tumour, as well as arteries supplying the normal colon, were dissected out at surgery from each patient and stored in cold isotonic saline solution. Similar mesenteric arteries were collected from 7 patients with no tumour pathology (4 with diverticulitis and 3 with Crohn's disease: mean age 44 $\pm$ 6 years, 3 males and 4 females, one of them a smoker) and likewise, the arteries were stored in cold isotonic saline solution. All arteries were used 3 to 12 hours after collection and once transported to the laboratory, the arteries were cut into 2 mm long segments and each segment was prepared for isometric tension recording in a 4-ml organ bath containing modified Krebs-Henseleit solution at 37°C (mM): NaCl, 115; KCl, 4.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; glucose, 11. The solution was equilibrated with 95% oxygen and 5% carbon dioxide to a pH of 7.3–7.4. Briefly, two fine steel wires 100 μm in diameter were passed through the lumen of the vascular segment, one wire fixed to the organ bath wall while the other was connected to a strain gauge for isometric tension recording (Universal Transducing Cell UC3 and Statham Microscale Accessory UL5, Statham Instruments, Inc.). This arrangement permits passive tension to be applied in a plane perpendicular to the long axis of the vascular cylinder. The changes in isometric force were recorded on a Macintosh computer using Chart v 3.6/s software and a MacLab/8e data acquisition system (ADInstruments). An optimal passive tension of 2 g was applied to the vascular segments and then they were allowed to equilibrate for 60–90 min. Before beginning the experiment, the vascular segments were stimulated with potassium chloride (50 mM) to determine the contractility of smooth muscle, and the segments which failed to contract at least 0.5 g were discarded.

Cumulative dose-response curves were recorded for endothelin-1 (10^{-10}–10^{-7} M) in the vascular segments from arteries supplying the tumour and from those supplying normal colon (control) in the same patients. To analyze the mechanisms involved in this response, the effect of endothelin-1 was recorded in the presence and absence of the nitric oxide synthase inhibitor, N^W-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M), the inhibitor of cyclo-oxygenase, meclofenamate (10^{-5} M), the endothelin ET_A receptor antagonist BQ123 (10^{-6} M), or the antagonist of endothelin ET_B receptors BQ788 (10^{-6} M). These antagonists were applied to the arteries 20 min before obtaining the dose-response curve for endothelin-1. Endothelium-dependent relaxation in response to bradykinin (10^{-9}–10^{-5} M) was also studied in tumour and control vascular segments precontracted with U46619 (10^{-7} M). The response to endothelin-1 and to bradykinin was also recorded in a group of mesenteric vascular segments from patients with no tumoural pathology (non-tumour). As the responses to endothelin-1 in these particular segments were similar to those in the control segments from the cancer patients, the blockers were not studied in the arteries from the patients with no tumour.

To study the expression of the endothelin ET_A and ET_B receptors, frozen tumour and control vascular segments (30–50 mg wet weight) were homogenized in ice-cold Tris buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma). After centrifugation (14,000 *g* for 15 min at 4°C), the protein concentration in the supernatant was determined with the BioRad DC protein assay kit (BioRad Laboratories). Equal amounts of protein were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were then transferred to PVDF membranes by wet electroblotting. The membranes were then blocked for 2 hours at room temperature in blocking buffer (Sigma) and

incubated overnight at 4°C with rabbit polyclonal antibody against endothelin ET_A or ET_B receptors (Santa Cruz Biotechnology) diluted 1:500. The membranes were then washed for 30 min, and further incubated with antirabbit IgG horseradish peroxidase conjugate (Amersham, 1:2000 dilution) at room temperature for 90 min, before the membranes were then washed again for 30 min. Immunolabelling was visualized by enhanced chemiluminescence (ECL) using the ECL reagent (Amersham) and the membranes were exposed to Hyperfilm (Amersham), which was scanned and quantified with Scion Image software.

Contraction in response to endothelin-1 was expressed as the percentage of the contraction produced by potassium, whereas the relaxation in response to bradykinin was expressed as the percentage of the active tone achieved with U46619, calculated as the mean ± standard error of the mean. The dose-response curves for each experimental condition was fitted to a sigmoid curve by non-linear regression, and the null-hypothesis was tested by comparing the sums-of-squares by the F test. The pD_2 of each dose-response curve was calculated as the negative logarithm of the concentration producing 50% of the maximal response by geometric interpolation. The pD_2 and the maximal response in tumour, control and non-tumour vascular segments, in the presence and absence of the blockers, were compared by one-way ANOVA followed by Dunnett's test to determine which comparisons were statistically significant. Protein expression data was normalised to the β-actin expression, which was determined on the same membranes using mouse monoclonal anti-β-actin antibody (Sigma). The expression of the distinct proteins was compared in tumour and control vascular segments using the unpaired Student's t test and a probability of less than 0.05 was considered as significant.

The compounds used here were: cyclo(D-Asp-Pro-D-Val-Leu-D-Trp) (BQ123); (2,6-dimethylpiperidinecarbonyl-γ-methyl-Leu-N_{in}-(methoxycarbony)-D-Trp-NIe-N-[N-[N[2,6-dymethyl-1-pyperidinyl) carbonyl]-4-methyl-L-leucyl]-1-(methoxycarbonyl)-D-tryptophyl]-D-norleucine sodium salt (BQ788); Nw-nitro-L-arginine methyl ester hydrochloride (L-NAME); meclofenamic acid sodium salt; bradykinin triacetate salt (all from Sigma); and the acetate salt of endothelin-1 (human, porcine, …, from Bachem, Switzerland).

Results

Stimulation with potassium chloride (50 mM) produced a similar contraction in arteries that were associated with tumours (2.6 ± 0.3 g) as in the control (2.2 ± 0.3 g) and non-tumour arteries (2.6 ± 0.4 g). After washing out the potassium chloride, endothelin-1 produced a dose-dependent arterial contraction that differed in the arteries associated with tumours and in the control arteries from the same patients ($P < 0.05$), as well as between tumour and non-tumour ($P < 0.01$) arteries. The maximal effect of endothelin-1 was similar in the three groups of arteries: tumour—194 ± 15%, 20 segments from 13 patients; control—215 ± 21%, 16 segments from 10 patients; non-tumour—233 ± 25 %, 10 segments from 4 patients. However, the sensitivity was higher in arteries associated with tumours ($pD_2 = 8.36 \pm 0.12$) than in control arteries ($pD_2 = 7.92 \pm 0.13$, $P < 0.05$) or non-tumour arteries ($pD_2 = 7.80 \pm 0.08$, $P < 0.01$).

Exposure to the nitric oxide inhibitor L-NAME or the cyclooxygenase inhibitor meclofenamate did not modify the response to endothelin-1 in any of the different types of tissue (Fig. 1). Similarly, in the segments precontracted with U46619, bradykinin (10^{-9}–10^{-5} M) produced a dose-dependent relaxation that was similar in all the three types of artery (Fig. 3).

By contrast, the ET_A antagonist BQ123 shifted the dose-response curves of endothelin-1 to the right ($P < 0.01$), significantly reducing the sensitivity to endothelin-1 in both control ($pD_2 = 7.45 \pm 0.05$ vs. 8.17 ± 0.26, $P < 0.05$) and tumour associated arteries ($pD_2 = 7.59 \pm 0.12$ vs. 8.54 ± 0.2, $P < 0.05$). The ET_B antagonist BQ788 also shifted the dose-response curve to the right ($P < 0.01$) and reduced the sensitivity of tumour associated arteries to endothelin-1 ($pD_2 = 8.07 \pm 0.08$ vs. 8.54 ± 0.2, $P > 0.05$) but not that of control arteries ($pD_2 = 8.29 \pm 0.22$ vs. 8.17 ± 0.26, Fig. 2). Despite this difference, both the ET_A (15 ± 4% of β-actin) and ET_B (30 ± 5% of β-actin) endothelin receptors, determined by immunoblotting, appeared to be expressed similarly in the control arteries (5 patients) and in the tumour associated arteries (20 ± 7% and 36 ± 5% for the ET_A and ET_B receptors, respectively, 5 patients) (Fig. 4).

Discussion

We found here that endothelin-1 produces a marked contraction of human mesenteric arteries, similar to that reported in studies of conduit human mesenteric arteries (12), and comparable to the results from blood vessels in other species such as the rat (13) or dog (14). We also found that arteries supplying colorectal tumours were more sensitive to this peptide (pD_2) than arteries from the same patients that supply blood to a region unaffected by the tumour, or those from patients with no tumour pathology. Since the contraction in response to potassium was similar in the three types of arterial tissue, the higher response to endothelin-1 in tumour arteries appears to be specific for this peptide and not due to increased contractility of the vascular smooth muscle. Although the patients with cancer were on average older (68 years) than the non-cancer patients (44 years), and they had higher vascular risk factors (7 patients with hypertension and 1 with diabetes mellitus), these differences were not likely to be the cause of the greater sensitivity to endothelin-1. Indeed, the arteries from these patients supplying normal colon tissue responded to endothelin-1 in a similar manner to the arteries from the patients not diagnosed with cancer, and less strongly than

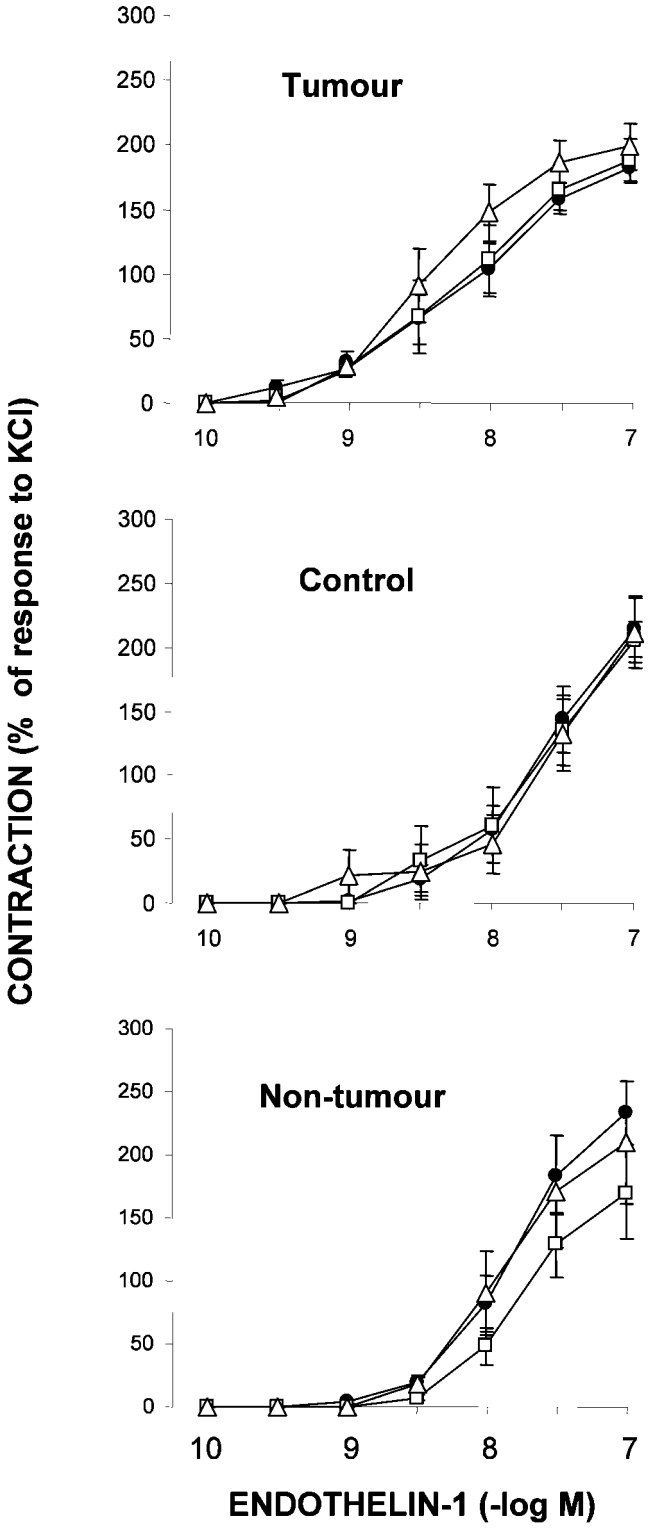

Figure 1. Contraction in response to endothelin-1 (10^{-10}–10^{-7} M) of mesenteric arteries supplying colorectal tumours (tumour), those supplying normal colon tissue in the same patients (control), or from patients with no tumour pathology (non-tumour), in the absence (●, control: 10 segments from 6 patients; tumour: 11 segments from 6 patients; non-tumour: 10 segments from 4 patients) or in the presence of L-NAME □; 10^{-4} M, control: 6 segments from 6 patients; tumour: 6 segments from 6 patients; non-tumour: 8 segments from 4 patients) or meclofenamate (△; 10^{-5} M, control: 6 segments from 6 patients; tumour: 6 segments from 6 patients; non-tumour: 8 segments from 4 patients). The data are the mean ± standard error of the mean.

the arteries from the tumour. These results contrast with those from an earlier study where blood vessels of a fibrosarcoma transplanted in rats responded weakly to endothelin-1 (15). Nevertheless, they are in agreement with the hyper-responsiveness to endothelin-1 observed in arterioles from liver tumours transplanted into mice (9). These discrepancies may be due to differences in the species, experimental procedure, and/or the type of tumour involved. The increase in the response found here may be due to local factors released by tumour cells affecting the neighbouring blood vessels. Indeed, cytokines such as TNF-α and interleukin-1β enhance contraction upon stimulation of endothelin ET_B receptors in arteries from humans (16) or rats (17, 18). While plasma concentrations of TNF-α may increase in patients with different tumours, they may vary from patient to patient, and local tumour tissue levels of this cytokine may be more pronounced than circulating levels (see 19). Accordingly, we found that the sensitivity to endothelin-1 increases in the arteries supplying the tumour but not in arteries supplying normal colon tissue in the same patients. Hence, this change may be induced by the local microenvironment of the tumour rather than by circulating systemic factors.

The contraction in response to endothelin-1 is mainly mediated by endothelin ET_A receptors in most normal tissues, although endothelin ET_B receptors may also participate in the response to this peptide in mesenteric arteries (20). Indeed, both receptor subtypes may be altered in tumour blood vessels. While stimulation of the endothelin ET_B receptor produces a stronger contraction in arteries from experimental tumours in rats (15), there are more endothelin ET_A receptors in experimental tumours in mice (9). Our findings suggest that the contraction produced by endothelin-1 is mainly mediated through endothelin ET_A receptors in normal human mesenteric arteries, as this contraction was blocked by the ET_A antagonist BQ123 and it remained unaffected by the ET_B antagonist BQ788. This partly agrees with the results from human omental arteries (21), where stronger inhibition of endothelin-1-induced contraction was obtained with ET_A and mixed ET_A and ET_B antagonists, than with the ET_B antagonist BQ788. However, while in that omental tissue BQ788 produced minor antagonism of the contraction to endothelin-1 (21), here it produced none at all. This may be due to the fact that this antagonist was used at a concentration one order of magnitude lower here (10^{-6} M) than in the earlier study (10^{-5} M, 21).

The stronger involvement of ET_A rather than ET_B receptors in the contractile response to endothelin-1 does not correlate with the expression of these receptors, as they were both expressed at similar levels. However, this response is in agreement with the situation in human omental arteries (21) in which there were similar levels of mRNA transcripts for each receptor, even though the contractile response was predominantly mediated by the ET_A subtype. Therefore, the ET_A receptors may be acting

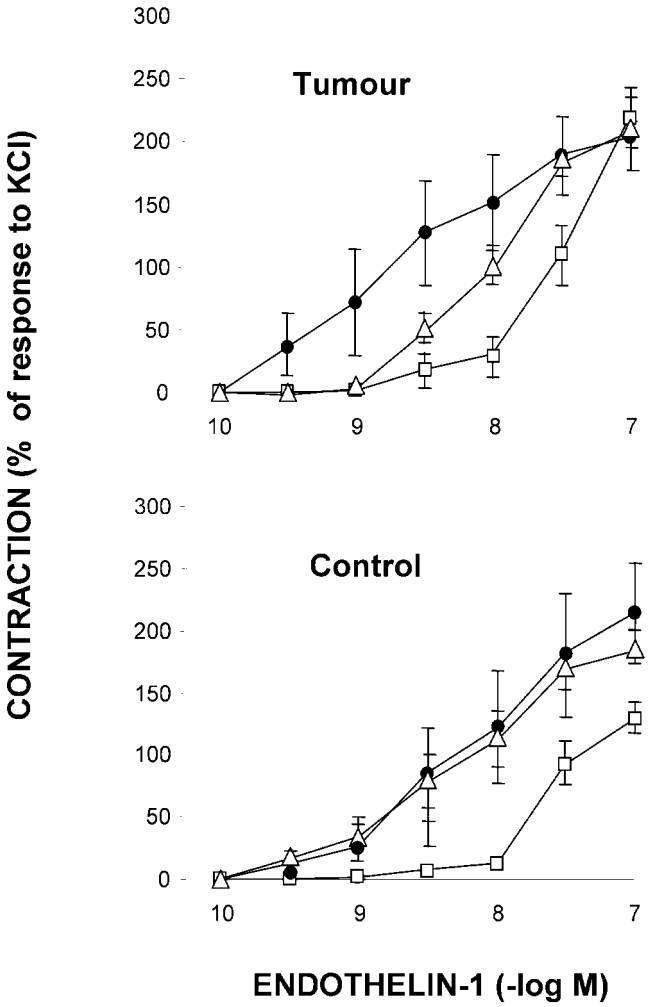

Figure 2. Contraction in response to endothelin-1 (10^{-10}–10^{-7} M) of mesenteric arteries supplying colorectal tumours (tumour), and those supplying normal colon tissue in the same patients (control), in the absence (●, control: 6 segments from 4 patients; tumour: 9 segments from 7 patients) or in the presence of BQ-123 (□; 10^{-6} M, control: 6 segments from 4 patients; tumour: 9 segments from 7 tumour patients) or BQ-788 (Δ; 10^{-6} M, control: 7 segments from 5 patients; tumour: 10 segments from 7 tumour patients). The data are the mean ± standard error of the mean.

more efficiently due to the downstream elements not shared with ET_B receptors.

In contrast to control arteries, both ET_A and ET_B receptors may be involved in the contraction to endothelin-1 in arteries supplying colorectal tumours since BQ123 and BQ788 both inhibited contraction. Therefore, in addition to endothelin ET_A receptors, vasoconstrictor endothelin ET_B receptors appear to be enhanced in the arteries supplying the tumour and they might be the cause of the increased sensitivity of these arteries to endothelin-1. In the non-vascular human colorectal cancer tissue the expression of endothelin ET_A receptors is augmented while that of ET_B receptors is reduced (22). However, to our knowledge the relative role of these two subtypes of endothelin receptors in the response to endothelin-1 of the arteries supplying human

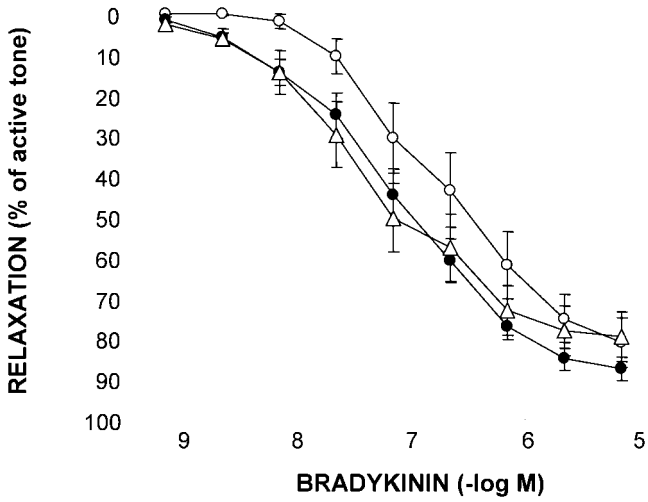

Figure 3. Relaxation in response to bradykinin (10^{-9}–10^{-5} M) of mesenteric arteries supplying colorectal tumours (●, tumour: 11 segments from 6 patients), those supplying normal colon tissue in the same patients (○, control: 7 segments from 4 patients), or from patients with no tumour pathology (Δ, non-tumour: 9 segments from 3 patients), precontracted with U46619 (10^{-7} M). The data are the mean ± standard error of the mean.

tumours has not previously been studied. As ET_A and ET_B receptor protein expression was not significantly different in control and tumour arteries, the enhancement of the ET_B-mediated response observed may be due to the potentiation of receptor affinity and/or through downstream effects.

In addition to endothelin-1 producing contraction by acting on vascular smooth muscle, there may be an interaction of endothelin-1 with other factors released by the endothelium, such as nitric oxide or prostanoids. This does not seem to be the case in human mesenteric arteries as the contraction induced by endothelin-1 was not modified by L-NAME or meclofenamate that modulate nitric oxide and prostanoids, respectively. Also, the relaxation to bradykinin was no different in mesenteric arteries supplying the tumour or from the normal colon, suggesting that tumour arteries exhibit normal endothelial function. Nevertheless, there are data showing that nitric oxide release may

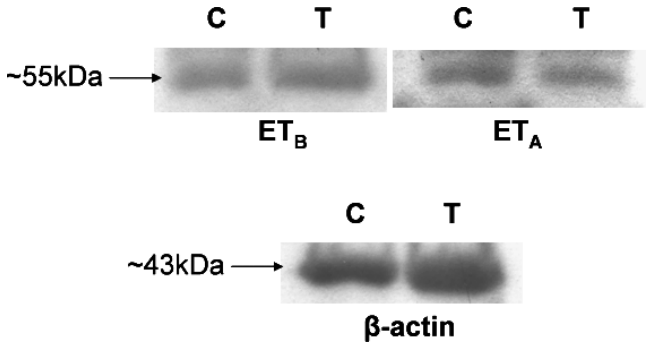

Figure 4. Representative western immunoblotting of ET_A endothelin receptors, ET_B endothelin receptors and β-actin in human mesenteric arteries irrigating a colorectal tumour (T) or normal colon (C).

be reduced in blood vessels associated with some experimental tumours (23).

In summary, our results suggest that arteries supplying blood flow to colorectal tumours may react more strongly to endothelin-1 when compared to arteries supplying normal colon tissue, probably due to an increase in the activity of endothelin ET_B receptors in those arteries. Significantly, these data may be of relevance for the treatment of these tumours, as antagonists of endothelin ET_B receptors may selectively increase blood flow and enhance the delivery of anti-cancer drugs to the tumour.

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