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Characterization and significance of ACE2 and Mas receptor in human colon adenocarcinoma

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Abstract

Introduction: A new arm of the renin–angiotensin system (RAS) has been recently characterized; this includes angiotensin converting enzyme (ACE)2 and angiotensin (Ang)1-7, a heptapeptide acting through the Mas receptor (MasR). Recent studies show that Ang1-7 has an antiproliferative action on lung adenocarcinoma cells. The aim of this study was to characterize RAS expression in human colon adenocarcinoma and to investigate whether Ang1-7 exerts an antiproliferative effect on human colon adenocarcinoma cells.

Materials and methods: Gene, protein expression and enzymatic activity of the main components of the RAS were determined on non-neoplastic colon mucosa as well as on the tumor mass and the mucosa taken 5 cm distant from it, both collected from patients with colon adenocarcinoma. Two different human colon cancer cell lines were treated with AngII and Ang1-7.

Results: The novel finding of this study was that MasR was significantly upregulated in colon adenocarcinoma compared with non-neoplastic colon mucosa, which showed little or no expression of it. ACE gene expression and enzymatic activity were also increased in the tumors. However, AngII and Ang1-7 did not have any pro-/antiproliferative effects in the cell lines studied.

Conclusions: The data suggest that upregulation of the MasR could be used as a diagnostic marker of colon adenocarcinoma.

Keywords

Angiotensin converting enzyme-2, angiotensin 1-7, cell cycle, cell lines, colon adenocarcinoma, Mas receptor, oncogene, renin–angiotensin system

Introduction

The renin–angiotensin system (RAS) consists of a group of enzymes and peptides in which, beside the classical arm, whose primary components are angiotensin converting enzyme (ACE) and angiotensin II (AngII), a recently discovered arm has been characterized. Particularly, the new arm of the RAS includes angiotensin converting enzyme 2 (ACE2), a carboxypeptidase that converts AngII to angiotensin 1-7 (Ang1-7), and Ang1-7, which is a peptide exerting opposite actions to those of AngII, through the interaction with its specific receptor, Mas receptor (MasR).[1] This new axis of the RAS has been reported to be expressed in a wide variety of cardiovascular and non-cardiovascular tissues, such as the duodenum, ileum, caecum and colon.[1]

Among all the experimental evidence on the effects exerted by Ang1-7, Tallant and colleagues showed the

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ability of this heptapeptide to inhibit the proliferation of vascular muscle cells as well as the angiogenesis observed in response to several growth factors.[2–4] Given these reports, Ang1-7 is now believed to serve as an endogenous regulator of cardiovascular cell growth.

Since then several studies have been carried out to investigate whether Ang1-7 might be considered a general regulator of cell growth beyond the vasculature. Overall, the data available so far have shown that Ang1-7 inhibits the proliferation of human lung cancer cells, by binding MasR,[5] and that Ang1-7 not only reduces the size of human lung tumor xenografts in vivo[6] but also markedly decreases their vessel density.[7]

Recently, the new RAS has been studied[8] in the setting of colorectal cancer (CRC), in which CRC liver metastases were found expressing significantly higher levels of MasR. However, the characterization of the new RAS (ACE2 and MasR) in the primary CRC tumor, as well as the evidence of an antiproliferative effect of Ang1-7 on it, is still lacking. Based on these observations, we aimed at evaluating the new RAS in human colon adenocarcinoma. For this purpose we decided to study in vivo the expression and the activity of the main components of the RAS in primary human colon adenocarcinoma as well as to determine in vitro any pro- and/or antiproliferative action of AngII and/or Ang1-7 in human colon adenocarcinoma cell lines.

Materials and methods

Selection of patients and sample collection

Consecutive patients, aged between 48 and 78 years, scheduled to undergo a surgical resection of the large bowel at the Department of General Surgery of AOUTS (Azienda Ospedaliera Universitaria di Trieste) because of colon adenocarcinoma ($n=33$), complicated diverticulitis ($n=2$) or megacolon ($n=1$), were recruited for this study. None had received chemotherapy or radiotherapy prior to surgical resection. Age and sex-matched consecutive patients scheduled to undergo a colonoscopy at the Gastroenterology Department of AOUTS, without history of colon adenocarcinoma and without macroscopic and/or microscopic evidence of this disease, were also recruited ($n=23$). These patients, along with those operated on for complicated diverticulitis and megacolon, were considered as controls ($n=26$). All patients were enrolled, after having signed informed consent, according to protocols approved by the Ethical Committee of the AOUTS.

Overall, 26 specimens of non-neoplastic colon mucosa (control group) as well as 33 specimens of colon adenocarcinoma (tumor group) and 33 specimens of apparently healthy mucosa taken 5 cm distant from the tumor border (tumor-free group) were collected and then either snap-frozen and stored at −70°C or fixed for histological analysis. For every patient, age, sex, and medications interfering with the RAS were recorded and, in the case of colon adenocarcinoma, site of sampling, grading and staging were also recorded.

Gene expression analysis

Three micrograms of total RNA extracted with Trizol (Invitrogen, Milan, Italy) from tumor ($n=33$) and tumor-free ($n=33$) specimens as well as their controls ($n=26$) was used to synthesize cDNA with Superscript First Strand synthesis system for reverse transcription–polymerase chain reaction (RT-PCR) (Gibco BRL, Invitrogen). ACE, ACE2, AT1 receptor (AT1R), C-reactive protein (CRP), inducible nitric oxide synthase (iNOS), MasR and tumor necrosis factor alpha (TNF-α) expression were analyzed by real-time quantitative RT-PCR using the TaqMan system based on real-time detection of accumulated fluorescence. Fluorescence for each cycle was quantitatively analyzed by an ABI Prism 7900HT Sequence Detection System (Perkin-Elmer Inc., Foster City, CA, USA). Gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18s ribosomal RNA. Primers and TaqMan probes were constructed with the help of Primer Express (Perkin-Elmer Inc.) (Table 1).

Immunohistochemistry

Four-micrometer paraffin embedded sections of colon adenocarcinoma and non-neoplastic colon mucosa were incubated for 45 min at 90°C in citrate buffer (pH 6) for detecting MasR. After neutralization of endogenous peroxidases, all the sections were incubated with a rabbit anti-MasR diluted 1:100 (Sigma Chemical, St Louis, MO, USA). Biotinylated immunoglobulins, diluted 1:400, were then applied as a secondary Ab. Specific staining was detected using the standard avidin–biotin complex method (ABC, Vector Laboratories, Burlingame, CA) and then visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical). Sequential sections were stained with hematoxylin and eosin to confirm the presence of adenocarcinoma. All the sections were examined by bright field microscopy (Olympus, BX50WI, Hamburg, Germany) and then digitized with a high-resolution camera (Q-imaging Fast 1394, Surrey BC, Canada).

ACE and ACE2 activity

Frozen specimens of tumor ($n=5$) and tumor-free areas ($n=5$) as well as their controls ($n=3$) were used for this analysis. Tissues were homogenized in ice-cold Tris-buffered saline (TBS: 25 mM Tris-HCl, 125 mM NaCl, pH 7.4), and homogenates pelleted by ultracentrifugation at 100,000g for 60 min at 4°C, resuspended in fresh TBS, and re-homogenized. Following a second ultracentrifugation step, the final membrane pellet was resuspended in 0.5 ml

Table 1. Sequences of probes and primers for the genes of interest

Gene of interest	Species		Sequences
ACE	Human	PROBE	6- FAM CCACCTGCTGGTCC
		F primer	GATCTACTCCACCGCCAAGGT
		R primer	GCGAGGAAGCCAGGATGTT
ACE2	Human	PROBE	6- FAM ACAGGCCAAGACATT
		F primer	GCTGCTCAGTCCACCATTGAG
		R primer	GCTTCGTGGTTAAACTTGTCCAA
AT1R	Human	PROBE	6- FAM AATGTAAGCTCATCCACC
		F primer	TTTCCTACCGCCCCTCAGA
		R primer	CAAAACATGGTGCAGGCTTCT
CRP	Human	PROBE	6- FAM CCAGCAGAGCAGATC
		F primer	CTGGAAAGGCCATTAGAATTGC
		R primer	TGCTTCATTTTGCTCTGGAAAA
iNOS	Human	PROBE	6- FAM AGCTGTGCATCGACCT
		F primer	CCTGCCAACGTGGAATTCA
		R primer	TCGAAGCGGCCGTACTTG
MasR	Human	PROBE	6- FAM ACCACCATGGAGTATGT
		F primer	CCCAAGTACCAGTCGGCATT
		R primer	GTCATTCCGAGAGTGACTCTCTTCT
TNF-α	Human	PROBE	6- FAM CCCATGTTGTAGCAAAC
		F primer	CTCGAACCCCGAGTGACAA
		R primer	GCTGCCCCTCAGCTTGAG

ACE: angiotensin converting enzyme, AT1R: AT1 receptor, CRP: C-reactive protein, iNOS: inducible nitric oxide synthase, MasR: Mas receptor, TNF: tumor necrosis factor

TBS, aliquoted, and frozen at −70°C. Prior to the activity assay, an aliquot of each sample was thawed on ice, diluted 10-fold in assay buffer (100 mM Tris, 1 M NaCl, pH 6.5), and protein was determined using a Pierce BCA Protein Assay Kit (Thermo Fischer Scientific, Illinois, USA).

Tissue ACE activity was measured using a modification of a previously published method.[9–11] Briefly, 5 µl of the membrane preparation was incubated at 37°C with the ACE substrate hippuryl-His-Leu (1 mM) in a total volume of 50 µl of buffer (0.4 M sodium borate buffer and 0.3 M NaCl, pH 8.3) in the presence and absence of EDTA (10 µM) for 30 min. Following incubation, 120 µl of 0.3 M NaOH and 10 µl of *o*-phthalaldehyde (20 mg/ml in methanol) were added. After 10 min at room temperature, 20 µl of 3 M HCl was added to stop the reaction, the tubes were centrifuged at 30,000 g for 5 min and the supernatants were transferred to a black 96-well microtiter plate. Fluorescence was measured using a FLUOstar Optima plate reader (BMG Labtechnologies). The rate of substrate cleavage was determined by comparison with a standard curve of the product His-Leu and is expressed as nmol of substrate/mg of protein/h.

For ACE2 activity, 100 µg of protein was incubated in duplicate with an ACE2-specific quenched fluorescent substrate (QFS) (7-methoxycoumarin-4-yl)-acetyl-Ala-Pro-Lys (2,4-dinitrophenyl) (Auspep, Victoria, Australia), 10 µM Z-Pro-prolinal (Auspep, Victoria, Australia), with or without 100 µM EDTA in a total volume of 200 µl.[12–14] Reactions proceeded at 37°C for 200 min with continuous monitoring of liberated fluorescence (λex = 320 nm, λex = 405 nm) using a FLUOstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany). The rate of substrate cleavage was determined by comparison with a standard curve of the free fluorophore MCA (4-amino-methoxycoumarin; Sigma) and is expressed as nmol of substrate cleaved/mg of protein/h.

Cell culture procedures

In this study two colon adenocarcinoma cell lines were considered: Caco-2 (continuous line of heterogeneous human epithelial colorectal adenocarcinoma cell line) and HT29 (human colon adenocarcinoma grade II cell line). Caco-2 cells were maintained in Eagle's Minimum Essential Medium (MEM) and HT29 in Dulbecco's modified Eagle's Medium (DMEM) (both media were purchased from Euroclone Ltd, Wetherby, West Yorkshire, UK). Both cell lines were grown in media containing 10% fetal bovine serum (FBS), penicillin (100 µg/ml), and streptomycin (100 U/ml); non-essential amino acids (Sigma Chemical) were added for culturing the Caco-2. Cell preparations were kept at 37°C in a humidified atmosphere of 5% CO_2.

Measurement of cell proliferation

Before the measurement of cell proliferation, the gene expression of MasR was determined in both Caco-2 and

HT29. Briefly, 3 µg of RNA extracted using Pure Link TM Micro-to-midi Total RNA Purification System (Invitrogen) was used for the cDNA synthesis and the gene expression of MasR was performed as previously described.

Confluent cell monolayers were removed from tissue culture flasks with trypsin and seeded into six-well tissue culture plates, with media containing 1% FBS. Caco-2 and HT29 cells were then treated for 24, 48 and 72 h with either Ang1-7 (10^{-7} M) or Ang II (10^{-7} M) (Sigma Chemical), which were replaced daily, due to their rapid degradation. The effect of Ang1-7 was also evaluated by treating the cells with three different doses of the peptide (10^{-7} M; 10^{-6} M; 10^{-5} M) for 72 h. The peptides were diluted in phosphate-buffered saline (PBS) before use and PBS alone was used in the controls. The dose and timepoints of the treatment were chosen according to what has been previously published.[5,15]

After the treatments, the cells were harvested with trypsin and fixed in 1 ml ice-cold 70% ethanol. The fixed cells were then resuspended in 1 ml of PBS containing 50 µg/l propidium iodide (Sigma Chemical) and 40 µg/l Rnase A (Sigma Chemical). Samples were analyzed on a Fluorescence Activated Cell Sorter (Coulter Elite ESP Cell Sorter, Beckman) measuring forward and site scatter, peak width, and area of fluorescence at 488 nm. Events were gated for peak width and area to exclude subcellular debris and aggregates. A minimum of 5000 gated events were recorded for each sample, and a frequency histogram of peak area was generated and analyzed using Modfit LT software.

Statistical analysis

The data were evaluated by one-way analysis of variance (ANOVA) calculated using Statview 512 software for Apple Macintosh computer (Brainpower, Calabasas, California, USA). We used χ^2 test and Fisher's least significant difference (LSD) method to determine statistically significant differences between group means. Results are expressed as mean ± SEM, unless otherwise specified. The criterion for statistical significance was $p < 0.05$.

Results

General characteristics of the patients studied

The patients did not differ in age (62.5 ± 6.5 years in the control group and 68.7 ± 5.3 years in the tumor/tumor-free group) or sex between the groups studied. A similar number of patients in each group were using medications affecting the RAS.

Expression of ACE, ACE2, AT1R, and MasR in colon adenocarcinoma and non-neoplastic colon mucosa

The gene expression analysis showed that MasR was significantly upregulated in both kinds of samples harvested from the patients with colon adenocarcinoma, being increased more than six-fold (*t*-test $p < 0.01$) and four-fold (*t*-test $p < 0.05$) in the tumor and tumor-free areas, respectively, compared with controls (Figure 1D). Moreover, the number of samples expressing MasR was significantly higher in the tumor group (χ^2 $p < 0.05$) compared with controls. These data were confirmed by immunohistochemistry, whereby we found a marked increase in the protein expression of MasR in the tumors whereas non-neoplastic mucosa showed little or no expression of this protein (Figure 2).

Of note, the tumors also showed an upregulation of ACE gene expression, even though to a lower extent than for MasR, since there was only a two-fold increase in gene expression compared with controls ($p < 0.05$) (Figure 1A). ACE2 and AT1R gene expression were unchanged among the groups studied (Figure 1B,C).

ACE and ACE2 enzymatic activity in human colon adenocarcinoma and non-neoplastic colon mucosa

Accordingly, ACE enzymatic activity was significantly increased in the tumors compared with colon mucosa ($p < 0.01$) (Figure 3A).

Although ACE2 activity was increased in the tumors studied, this did not reach significance (Figure 3B).

To better interpret these data, we decided to evaluate the gene expression of molecules that have been demonstrated to be increased in inflammatory bowel diseases,[16] such as CRP, iNOS and TNF-α. Whereas the gene expression of TNF-α was unchanged among the groups studied, CRP and iNOS were increased in both the tumors and the tumor-free areas with respect to the control. In particular, for CRP, there was a 15-fold increase in its gene expression compared with controls ($p < 0.05$), while for iNOS there was a four-fold increase, which, however, was not significant.

Effects of AngII and Ang1-7 on cell cycle events in human colon adenocarcinoma cells

The effects of AngII and Ang1-7 were evaluated in two different colon adenocarcinoma cell lines, in which the expression of MasR had been previously confirmed. Of note, in the Caco-2 cells the gene expression of MasR was three-fold higher than in the HT-29 cells. Neither treatment had

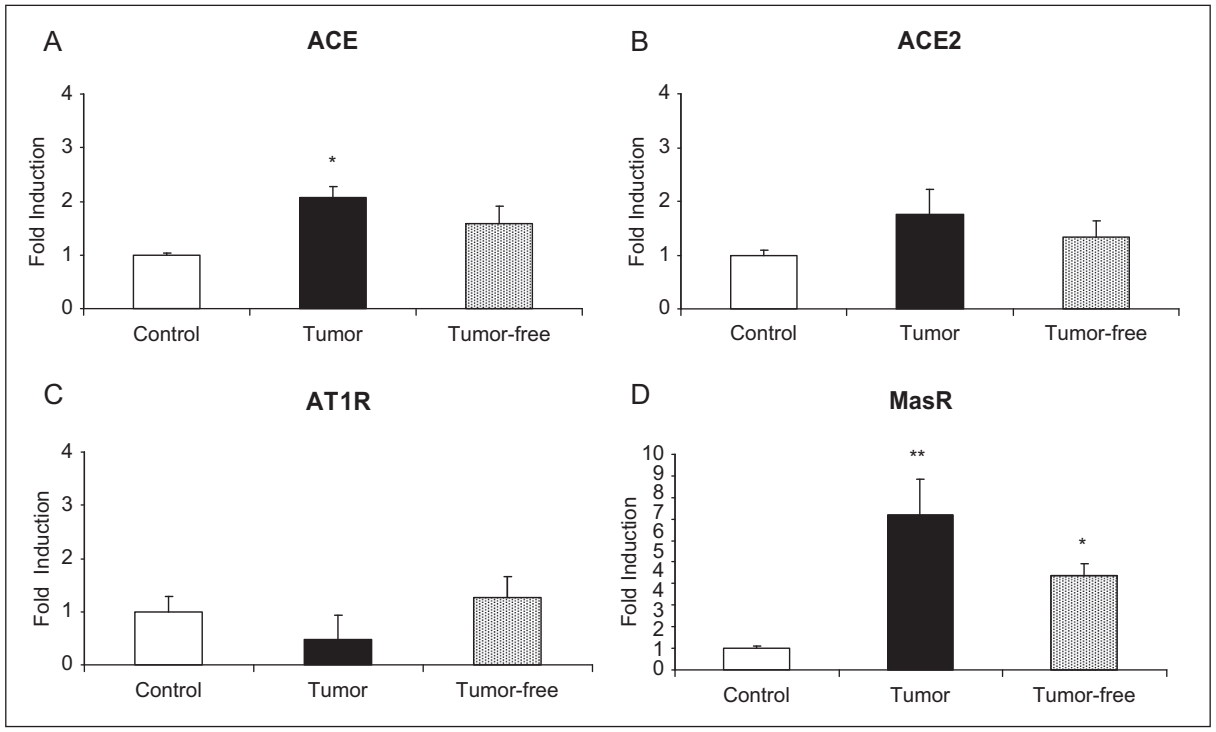

Figure 1. ACE (A), ACE2 (B), AT1R (C), and MasR (D) gene expression. The gene expression (mRNA) was tested on non-neoplastic mucous membrane (Control; $n = 26$), human colon adenocarcinoma (Tumor; $n = 33$), neoplastic mucous membrane 5 cm distant from the mass (Tumor-free; $n = 33$). mRNA expression is reported as relative gene units; data is expressed as mean ± SEM. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control.
ACE: angiotensin converting enzyme, AT1R: AT1 receptor, MasR: Mas receptor.

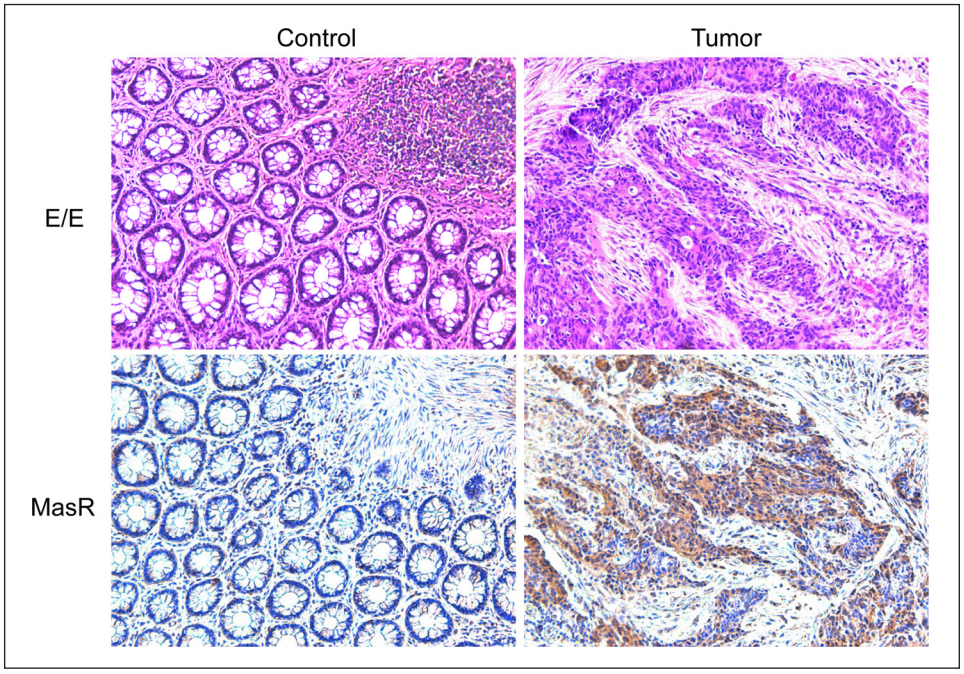

Figure 2. Representative photomicrographs of non-neoplastic colon mucosa and colon adenocarcinoma showing the protein expression of Mas receptor (MasR) within the neoplastic mass. Hematoxylin eosin (E/E) on non-neoplastic colon mucosa (Control) and colon adenocarcinoma (Tumor) 10× magnitude. Immunohistochemistry for MasR on non-neoplastic colon mucosa (Control) and colon adenocarcinoma (Tumor) 10× magnitude.

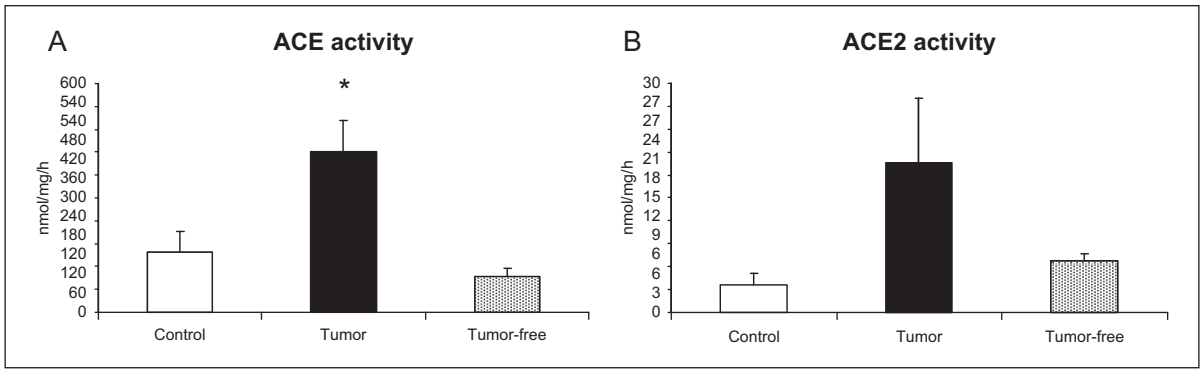

Figure 3. ACE (A) and ACE2 (B) enzymatic activity. The enzymatic activity was tested on non-neoplastic mucosa (Control; $n = 3$), human colon adenocarcinoma (Tumor; $n = 5$), neoplastic mucosa 5 cm distant from the tumor mass (Tumor-free; $n = 5$). Enzymatic activity is reported as nmol/mg/h; data are expressed as mean ± SEM. *$p < 0.01$ vs. tumor-free.
ACE: angiotensin converting enzyme.

an effect on the cell cycle events in either cell line at the different timepoints (Figure 4) and doses studied.

Discussion

Recently, the classical view of the RAS has been challenged by the discovery of ACE2 and its products. ACE2 is a carboxypeptidase that cleaves AngII to generate Ang1-7, which exerts its effects through the MasR. ACE2 has been shown to be expressed in cardiovascular as well as in a wide variety of non-cardiovascular tissues, such as the ileum, duodenum, jejunum, caecum, and colon.[1] Data from in vitro experiments have also demonstrated that Ang1-7 has an antiproliferative effect. In this work, to characterize the new RAS in human colon adenocarcinoma, we have determined the expression and the activity of the main components of the RAS in primary human colon adenocarcinoma as well as the effects that AngII and/or Ang1-7 have on cell cycle events in human colon adenocarcinoma cell lines.

Firstly, our results demonstrate that ACE gene expression (Figure 1A) and ACE activity (Figure 3A) increased significantly in the tumors. On the other hand, ACE2 activity increased, but not significantly, which might have been due to its high variability between the samples analyzed but also to the limited number of samples analyzed (Figure 3B).

The increase in the enzymatic activity of ACE in colon adenocarcinomas could be correlated with the local increase in proinflammatory molecules, since it has been demonstrated that inflammation modulates the RAS.[15] A certain degree of inflammation is expected to be present in tumors;[10,16] thus the analysis of CRP and iNOS demonstrated a significant increase in the gene expression of these inflammation-related molecules in tumors.

Despite the limited number and type of cell lines studied, the treatment with AngII did not affect the cell cycle events in human colon adenocarcinoma cells, suggesting that any increase in ACE activity, and thus AngII levels, should not have any effect on cell proliferation (Figure 4). A further analysis extending to other cell lines of human colon adenocarcinoma could be useful to fully analyze the effect of AngII in this setting.

Secondly, the novel finding of this work is that MasR was significantly upregulated in colon adenocarcinoma compared with non-neoplastic colon mucosa. Of note, we found that the tumors were expressing significantly higher levels of MasR compared with the controls (t-test $p < 0.01$) and that the number of tumors expressing MasR was also significantly higher compared with the controls (χ^2 $p < 0.05$) (Figure 1D). This was confirmed by immunohistochemistry, whereby a high expression of MasR could indeed be visualized within colon adenocarcinomas, whereas there was little or no expression in the non-neoplastic controls (Figure 2).

The argument for an increase in the expression of the MasR within neoplastic masses has already been made by Neo and colleagues,[8] who found a higher expression of this molecule in hepatic colorectal (CRC) metastases compared with the surrounding liver tissue. Of note, here, for the first time, we report a significant induction of MasR within primitive human colon adenocarcinomas.

This finding is consistent with the fact that MasR, before being identified as the functional ligand for Ang1-7, had been cloned and sequenced as a human oncogene, by a cotransfection and tumorigenicity assay.[17] In particular, this was an assay that aimed to detect cellular oncogenes by transfection of NIH 3T3 cells with genomic DNAs isolated from a wide variety of human malignancies. Using this method, nude mice transfected with NIH 3T3 cells, which had been previously cotransfected with DNA from human epidermoid carcinoma, developed different tumors, which then led to the identification of the MasR sequence. Later on, nude mice transfected with cells carrying the MasR DNA sequence developed tumors within 2 weeks after the injection, and similarly NIH 3T3 cells transfected with MasR DNA formed foci within 16 days.[18] The same authors identified the DNA rearrangement in the 5′ noncoding sequence as responsible for the activation of the MasR oncogene.[18]

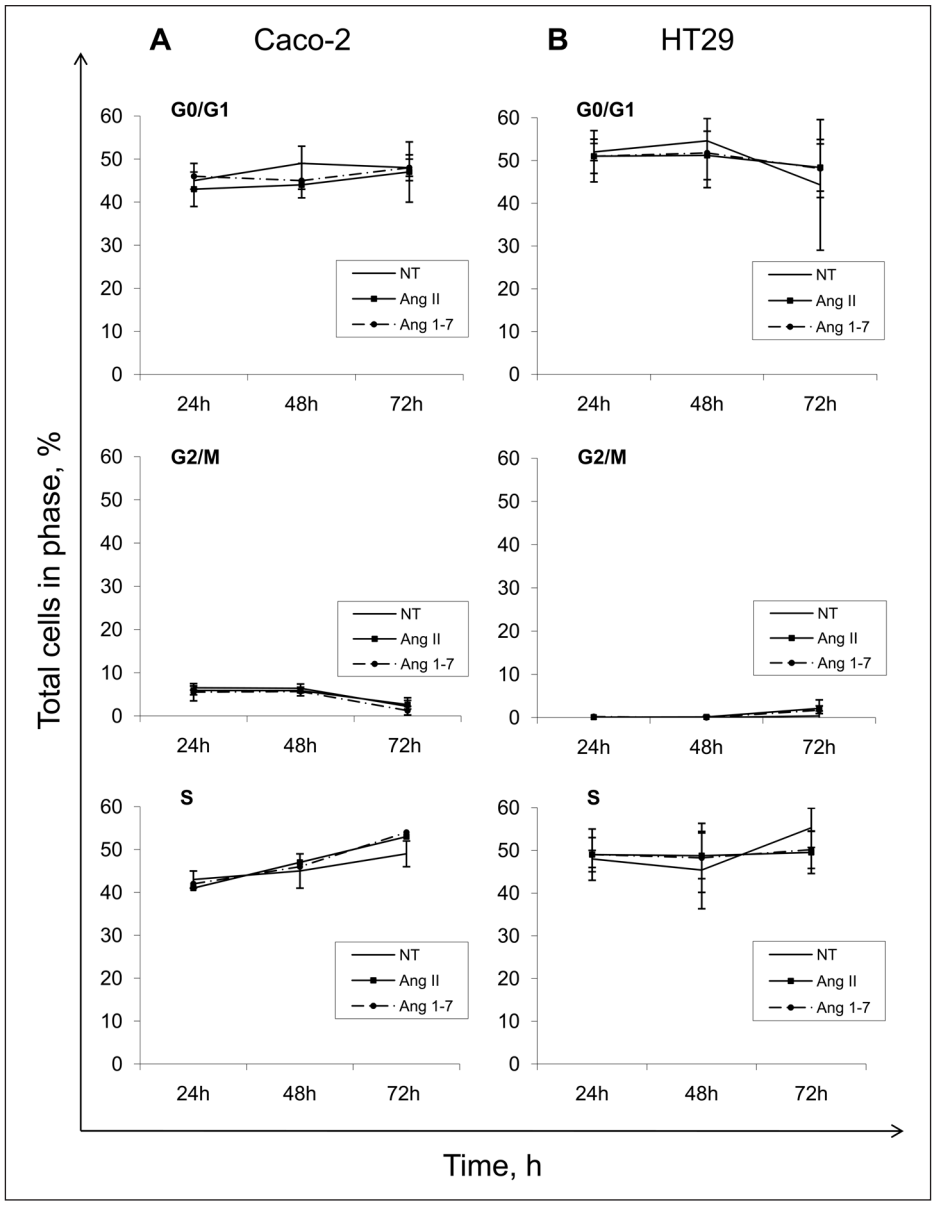

Figure 4. Flow cytometry analysis of cell cycle events (G0/G1; G2/M; S) after incubation with PBS alone (non-treated cells; NT), Ang1-7 ($^{-7}$ M), Ang II ($^{-7}$ M) for 24, 48 and 72 h. (**A**) Caco-2, (**B**) HT29. Results are expressed as mean ± SD of two independent experiments.

Activation of oncogenes, which occurs by chromosomal rearrangements, mutations, and gene amplification, confers a growth advantage or increased survival on cells carrying such alterations. These phenomena are of considerable importance to developing rational cancer treatments, since oncogenes are involved in cancer initiation and progression (as when chronic myelogenous leukemia converts to acute leukemia) and they may also be used as therapeutic targets.[19]

Thus, since MasR activation by Ang1-7 seems to mediate a number of potentially anti-angiogenic and anti-proliferative effects,[2–4] to test whether increased MasR expression could be used as a target for initiating anti-tumor responses, we treated two different cell lines of human colon adenocarcinoma, which were both expressing MasR, with Ang1-7. The method we followed was the same that had previously led to a reduced proliferation of human lung adenocarcinoma cells after Ang1-7 treatment. However, Ang1-7 treatment was not associated with any change in the cell cycle events (Figure 4). This might be explained by the different responsiveness to this peptide that has been observed in different cell types. For instance, although Ang1-7 decreased the proliferation in several cell types, it also promoted cellular proliferation in the hair follicles at the edge of the wound and hematopoietic progenitors.[20,21] In addition, as for AngII, further studies on other human colon adenocarcinoma cell lines should be performed to fully evaluate the effect of Ang1-7 on cell cycle events in colon adenocarcinoma.

Anyway, to link our data to the current literature, even though MasR is highly expressed in colon adenocarcinoma, it does not seem to be involved in any oncogenic pathway leading to cancer development. As a matter of fact, transgenic mice overexpressing Ang1-7 do not show increased tumor formation, and MasR overexpression in the retina leads to increased cell death without tumorigenicity, suggesting that MasR itself is not oncogenic.[22]

Overall, our work, together with the experimental evidence available so far, suggests that the Mas receptor, whose expression significantly increases within a tumoral mass, would warrant further investigations to be used as a diagnostic marker of colon adenocarcinoma. Thus, further studies evaluating whether MasR is increased in the early stages of colon adenocarcinoma are needed to determine the value of MasR as a diagnostic tool.

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Conflict of interest

The authors declare that there is no conflict of interest.

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