Aberrant expression of OX1 receptors for orexins in colon cancers and liver metastases: an openable gate to apoptosis

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/)

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Abstract

Resistance to apoptosis is a recurrent theme in colon cancer. We have shown previously that the 7-transmembrane spanning receptor OX1R for orexins promote robust apoptosis in the human colon cancer cell line HT29 through an entirely novel mechanism involving phosphorylation of tyrosine-based motifs in OX1R. Here, we investigated the status of OX1R in a large series of human colorectal tumors and hepatic metastases. All primary colorectal tumors regardless of their localization and Dukes’ stages and all hepatic metastases tested expressed OX1R mRNA and/or protein. In sharp contrast, adjacent normal colonocytes or hepatocytes as well as control normal tissues were negative. Next, we showed that 9 human colon cancer cell lines established from primary tumors or metastases expressed OX1R mRNA and underwent important apoptosis upon orexin-A challenge. Most interestingly, orexin-A also promoted robust apoptosis in cells which are resistant to the most commonly used drug in colon cancer chemotherapy, 5-fluorouracil. When human colon cancer cells were xenografted in nude mice, orexin-A administered at day 0 strongly slowed the tumor growth and even reversed the development of established tumors when administered 7 days after cell inoculation. Orexin-A also acts by promoting tumor apoptosis in vivo since caspase-3 is activated in tumors upon orexin treatment of nude mice. These findings support that OX1R is an Achilles heel of colon cancers, even after metastasis or chemoresistance. They suggest that OX1R agonists might be novel candidates for colon cancer therapy.
Introduction

Colon cancer is a leading cause of cancer mortality in many countries (1). Molecular genetic studies have identified key genes whose mutations or altered expression can cause colon cancer (2). Many observations also indicated that colon cancer growth is under the control of a variety of peptide growth factors (3) acting at tyrosine kinase receptors or G protein-coupled receptors. Among those receptors, epidermal growth factor receptor (EGFR) is prominent in colon cancers (4) due to its autoactivity and also because it can be transactivated by G protein-coupled receptors (5). It is a therapeutic target since antibodies directed against EGFR have shown efficacy in colorectal cancer (4).

Much less is known about receptors and/or peptides that inhibit growth and/or promote apoptosis of colon cancer cells. Some years ago we reasoned that besides growth factors or peptide hormones that promote colon cancer growth (3), we should be able to find natural peptides behaving as suppressors of colon cancer growth. We tested this hypothesis by screening the ability of a large series of peptide hormones and neuropeptides to inhibit the growth of the human colon cancer cell line HT29 cultured in the presence of fetal calf serum (6). We made two hits with orexin-A and orexin-B which appear to be robust growth inhibitors (6). Orexins do not alter cell cycle but promote strong apoptosis by inducing cytochrome c release from mitochondria to cytosol and activation of caspase-3 and caspase-7 (6).

Orexin-A and orexin-B (7), also named hypocretin-1 and hypocretin-2 (8), were discovered as hypothalamic peptides that are encoded in a preproorexin precursor. The two peptides are present in hypothalamic neurons that regulate sleep, wakefullness, feeding, breathing, reward system or drug addiction (9). Functions of orexins have been also described in a few peripheral tissues (10, 11) but expression
of orexins at the periphery remains debatable (11-13). Classically, the actions of orexins are mediated by two seven-pass transmembrane receptors OX1R and OX2R (7) the activation of which induces cellular calcium transients through Gq-dependent and -independent pathways (14).

We showed that OX1R triggers apoptosis by an entirely novel mechanism (15, 16) which is not related to Gq-mediated phopholipase C activation and cellular calcium transients (13). Orexins induce tyrosine phosphorylation of two tyrosine-based motifs in OX1R, ITIM and ITSM, resulting in the recruitment of the phosphotyrosine phosphatase SHP-2 the activation of which is responsible for mitochondrial apoptosis (15, 16).

Given that i) failure of tumor cells to undergo apoptosis translates into tumor progression and chemotherapeutic resistance, ii) apoptosis emerges as a potential target for cancer treatment (17, 18), it was important to determine the status of orexin receptors and orexin-induced apoptosis in colon cancer. In the present work, we addressed this question by studying the expression of orexin receptors in a large series of primary colonic tumors and also in hepatic metastases. We also investigated the ability of orexins to promote apoptosis in a large panel of colon cancer cell lines. We show that the receptor OX1R is not present in normal colon or liver but is aberrantly expressed in all primary tumors and hepatic metastases tested. Futhermore we demonstrate that orexins promote apoptosis in most of the colon cancer cell lines tested including in cells which are resistant to 5-fluorouracil, the most commonly used drug in colon cancer chemotherapy (4). Finally, we showed that orexins strongly reduced the development of tumors in nude mice xenografted with human colon cancer cells. Since peripheral expression of orexins is debatable (13), we also studied the expression of the orexin precursor mRNA by RT-qPCR in primary
colon tumors, hepatic metastases and normal colon and liver samples. No orexin precursor mRNA could be detected in any samples. These data indicate that OX1R may be considered as a new valuable gate to apoptosis which could be openable by exogenous orexins or OX1R agonists in colon cancer even after metastasis and chemoresistance to 5-fluorouracil.
Materials and Methods

Tissue collection

Thirty-eight archival specimens of formalin-fixed, paraffin-embedded human colonic carcinomas were selected to offer a sampling according to the severity of the disease and the different sites of the large intestine (see Table 1). Biopsy samples obtained during endoscopy of normal adults suffering from irritable bowel syndrome were also studied. Several frozen unfixed tissues were used for RT-qPCR: 10 colonic carcinoma, 5 non tumoral colon tissues, 10 hepatic metastasectomies and 7 non tumoral hepatic tissues. Tissues were used in accordance with the requirements of the Human Research Committee of Hospital Bichat, Paris. Hypothalamus cDNAs prepared from human normal brains were obtained from Clontech (St Quentin en Yvelyne, France).

Cell Lines culture

The human colon cancer cell lines were obtained from ATCC (Rockville, MD). The cell lines were established from resected tumors (19, 20) as follows: HT29, SW480, SW48, Caco-2, LS174T and HCT116 from primary colorectal adenocarcinomas; T84 from lung metastases; SW620 from lymph node metastases; Colo205 from ascite metastases and LoVo from left supraclavicular metastases. The HT29-FU cell line which resists to 1 µM 5-fluorouracil (5-FU) was obtained as described (21, 22). Cell lines were biannually verified by morphology, growth characteristics and to be mycoplasma-free (PCR). Cells were routinely cultured in 25 cm² plastic flasks (Costar, Cambridge, MA) and maintained at 37°C in humidified atmosphere of 5% CO₂/air in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g glucose/L, supplemented with 10% FCS (Invitrogen™, Cergy
Pontoise, France). Caco-2 cells were grown in DMEM with 20% FCS and 1% nonessential amino acids. Colo205 cells were grown in RPMI 1640, LoVo cells in Ham’s F12 and T84 cells in 50% DMEM/50% Ham’s F12 supplemented with 10% FCS.

**Immunohistochemical procedures**

Five µm thick sections were incubated at 37°C with a rabbit polyclonal antibody raised against human OX1R (OX1R11-A) (Alpha Diagnostic International, San Antonio, TX, USA) diluted 1:50 (20 µg/ml). Thereafter, sections were incubated with biotinylated secondary antibody diluted 1:200, and finally with the avidin-biotin complex diluted 1:100 (Kit ABC Vectastain; Vector Laboratories, Burlingame, CA). The peroxidase activity was revealed by the diaminobenzidine and nuclei were counterstained with Mayer’s hemalum. Negative controls were obtained by omitting the primary antibody, by replacing the latter by normal rabbit IgG or by preincubation of the primary antibody with the homologous immunogen peptide (OX1R11-P, Alpha Diagnostic International, San Antonio, TX, USA). It was also verified that 50 µg of the hOX1R immunogen peptide per ml of diluted antiserum totally abolished the signal. In separate experiments, we have demonstrated that the anti-OX1R antibody stains CHO cells expressing the recombinant OX1R whereas no staining could be observed in parental non transfected CHO cells (not shown). The intensity of immunostaining was evaluated on three criteria: the estimation of the percentage of immunoreactive cells on the totality of the section; the intensity of the immunoreactivity expressed as negative, -, discrete to weak and moderate, +, strong, ++ or intense, +++ ; and the identification of the cellular components which expressed the immunoreactivity.
Quantification of apoptotic cells by annexin V labelling

Apoptotic cells were determined using the Guava Nexin™ kit (Guava Technologies, Hayward, CA, USA) which discriminates between apoptotic and non-apoptotic cells as described (6). Cells were incubated with fresh medium containing or not 1 µM orexin-A. After 48 h, apoptotic cell staining was analyzed with a Guava PCA system (15). Results are expressed as the percentage of apoptotic Annexin V-PE positive cells and are the means of 3 independent analyses.

RT-qPCR procedure

Total RNA (RNA_T) in cultured cells and in frozen unfixed tissue sections were extracted using NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany). Quality and integrity of RNA were evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Real-time PCR amplification was performed using a LightCycler® 480 PCR system (Roche Diagnostics, Meylan, France). The qPCR medium included 4 µl of cDNA, 10 µl of Light Cycler 480 SYBR Green I master mix (Roche Diagnostics, Meylan, France) and 2 µl of each forward and reverse primer in a final reaction volume of 20 µl. Primer sets (QuantiTect® Primer Assays) were designed against the complete nucleotide sequences, as deposited on GENE GLOBE, using Qiagen QT00058912 (Hs_HCRTR1_1_SG) for the human OX1R, QT02288356 (Hs_HCRTR2_2_SG) for the human OX2R and QT01192646, (Hs_GAPDH_2_SG) for the calibrator GAPDH. For prepro-orexin, the primers were 5’-CCAAGCACCATGAACCTCC-3’ (sense) and 5’-GCAAGAGCAAGTCTTTTGACG-3’ (antisense). Assays were run using the following protocol: 95°C for 5 min, 95°C for 10 sec, gene specific annealing temperature 60°C for 30 sec, 72°C for 30 min for 40 cycles, followed by a gradual increase in temperature from 50°C to 97°C during the
melting stage. The OX1R, OX2R, preproorexin and GAPDH amplicon sizes were 85 bp, 103 bp, 148 bp and 119 bp, respectively. C\textsubscript{t} (threshold cycle value) values were converted into quantities relative (Q\textsubscript{rel}) and corrected for PCR amplification efficiency, using the following formula: \( Q\textsubscript{rel} = 2^{-[\Delta C(t(sample)-\Delta C(t(sample))]}. \)

**Tumorigenicity assay in nude mice**

Exponentially growing LoVo, HT29 or HCT116 cells were harvested, washed with PBS and resuspended at a concentration of \( 1 \times 10^{7}/\text{ml} \). Mice were anesthetized by i.p. injection of Rompun 2%, Clorketam 1000 in 140 \( \mu \text{l} \) of 0.9% (w/v) NaCl. Cells (\( 10^{6}/100 \, \mu \text{l} \)) were then inoculated s.c. into the flank of mice. All nude mice developed tumors following injection of \( 10^{6} \) LoVo, HT29 or HCT116 cells. Tumor development was followed by caliper measurements in 2 dimensions (L, W), and the volume (V) of the tumor was calculated by the formula for a prolate ellipsoid (\( V = L \times W^{2} \times 3.14/6 \)) as reported (23). For treatment with orexin-A, the peptide was dissolved at a concentration of 1 mg/ml in PBS and 0.112, 1.12 or 11.2 \( \mu \text{moles/kg of body weight} \) were administered daily by i.p injection. Control mice received PBS. The body weight of mice was similar after 30 days of treatment with and without orexine-A, i.e. 24.7 \pm 1.4 (n = 6) and 23.2 \pm 0.6 (n = 6), respectively. No adverse effect of orexin could be observed during treatment. Cleaved caspase 3 (C92 605, BD Pharmingen, Rungis, France, 1:200 dilution) expression was studied by immunohistochemistry on selected paraffin sections of tumors developed in nude mice (24).
Results

**OX1R is aberrantly expressed in human colon tumors ex vivo.**

We investigated OX1R expression in colonic adenocarcinomas versus normal colonic epithelium by immunohistochemistry. All the 38 tumors tested (Table 1) were immunoreactive for OX1R (Figure 1). The immunostaining was specific since it disappeared after incubation of the OX1R antibody with its homologous immunogen peptide (Figure 1) or when the primary antibody was omitted (not shown). On sections, 50 to 100% of cells were immunoreactive (Table 1). In contrast, normal proximal and distal mucosae of patients with irritable bowel syndrome, taken as controls, did not express OX1R (Figure 1A). This is in good agreement with RT-qPCR experiments which also failed to detect any OX1R mRNA in the same tissue samples (Table 2). The mean percentages of immunoreactive cells were similar in the proximal and distal colon i.e. 82 and 83%, respectively. In these two sites, there was no correlation between the numbers of cells showing OX1R expression and the severity of the tumor. The same holds true for the intensity of immunostaining which was also independent of the severity of the tumor, variable from one tumor to another and between cells within a tumor. Immunostaining was observed in neoplastic glands whereas normal glands and still normal colonocytes remained negative (Figure 1C, D). OX2R mRNA was not detectable by RT-qPCR in any colon cancer tested (Table 2). We conclude that OX1R is aberrantly expressed in epithelial cells during colon carcinogenesis.

The immunoexpression of OX1R in colon cancer cells was detected predominantly intracellularly in cytoplasm and/or nucleus but also at the plasma membrane with a variability depending on the tumor (Figure 1). For the whole of colonic cancers, the immunoreactivity predominated for 58% of them in the
cytoplasm, 47% in nuclei, whereas membranes were seen in 34%. Dysplastic mucosae neighbouring cancers showed a variable immunolabeling. Since the main paradigm of seven transmembrane spanning receptors (25) including orexin receptors (13) is activation at the plasma membrane by external ligands, we further explored the cellular distribution of OX1R in HT29 cells in which external orexins promote apoptosis (6). When immunostaining was performed on HT29 cell pellets under the same procedure as that used for colon tumors, very similar data were obtained i.e. labelling of intracellular compartments and the plasma membrane (Supplementary Fig.1).

Since orexins were previously claimed to be present in the gut (26), though this view is now debatable (13), we tested the possible presence of the orexin precursor mRNA in 10 colonic tumors. No orexin precursor transcript could be detected in any colon tumor (Table 2) under conditions in which specific amplification products were detected in human hypothalamus taken as a control tissue where orexins are mainly produced (7, 8). These data indicate that colon tumors express the orexin receptor OX1R but not the orexin peptides.

**OX1R is aberrantly expressed in human hepatic metastases from colorectal cancers ex vivo**

Next, we raised the question of whether OX1R present in primary colon tumors were still expressed after migration in their main site of metastasis, e.g. the liver. A collection of 10 human hepatic metastases was investigated for the expression of OX1R transcripts using RT-qPCR. Seven samples of normal liver tissue which are adjacent to metastases were used as controls. As shown in the Figure 2A, expression of OX1R mRNA was obtained in all hepatic metastases. In contrast, RT-
qPCR experiments failed to detect any OX1R mRNA in normal liver samples (Figure 2A). The expression of OX1R mRNA was similar in liver metastases and primary colon tumors (Figure 2 and Table 2). We confirmed these data by investigating OX1R expression by immunohistochemistry in hepatic metastases (Figure 2B). All metastatic tumors studied expressed OX1R whereas adjacent normal hepatocytes remained negative. It is also worth noting that no orexin precursor mRNA could be detected by RT-qPCR in human liver (Table 2). Altogether these data indicated that after colon cancer cell migration to the liver, metastatic nests still express OX1R.

OX1R mRNA expression and orexin-induced apoptosis in human colon cancer cell lines including 5-FU-resistant cells

A large collection of colon cancer cell lines was investigated for the expression of OX1R transcripts using RT-qPCR. Expression of OX1R mRNA could be detected in all colon cancer cell lines but one, i.e. HCT116 (Figure 3A). No mRNA for the other orexin receptor subtype OX2R could be detected in any cell line tested (not shown). Orexin-A (1 µM) induced apoptosis (Figure 3B) and subsequent reduction in cell number (Supplementary Fig. 2) in all colon cancer cell lines tested but one, i.e. HCT116. Similar results were obtained with orexin-B (data not shown). It is worth noting that these effects were observed in culture medium containing FCS which promotes robust cell growth. The extents of apoptosis were variable from one cell line to another (Figure 3). Quite interestingly, they are strongly correlated with the amounts of OX1R mRNA detected in the various cell lines (Figure 3C). Altogether, these data indicated that functional OX1 receptors that are able to promote apoptosis are readily expressed in most of the colon cancer cell lines.
The most commonly used drug in colon cancer chemotherapy, 5-fluorouracil (5-FU), is able to induce apoptosis in colon cancer cells but development of drug resistance is a primary cause of failure of chemotherapy (4). To investigate the functionality of OX1R in 5-FU-resistant colon cancer cells, we used the HT29-FU cell line in which a long-term 5-FU exposure selected cells resistant to the drug (21). The expression of OX1R mRNA was similar in HT29-FU cells and parental HT29 cells (Figure 3A). We verified that 5-FU up to 1 µM did not promote apoptosis or reduction in cell number (Supplementary Fig.3) in HT29-FU cells whereas it was quite active in the parental HT29 cells (Figure 4). Orexin-A was able to induce strong apoptosis in the HT29-FU cells which are resistant to 5-FU action (Figure 4). When we considered the parental HT29 cells which are sensitive to 5-FU (21), it was also very interesting to observe that orexin-A induced apoptosis not only in the absence of 5-FU (see Figure 3A and Figure 4) but also in the presence of 1 or 10 µM 5-FU (Figure 4). Similar data were obtained with orexin-B (not shown). These data show that orexins promote apoptosis in 5-FU-resistant cells.

Effect orexin-A on the growth of tumors developed by xenograft of colon cancer cells in nude mice

Subcutaneous inoculation of 10^6 LoVo cells into the flank of nude mice resulted in the development of tumors at the site of inoculation (Figure 5). Tumor development was followed until 15 or 30 days and autopsy of mice did not revealed any metastatic site. Daily i.p. injection of orexin-A (1.12 µmoles/Kg) beginning the day colon cancer cells were xenografted into mice resulted in strong decrease of tumor volumes in LoVo cells. As for control mice, no metastatic site of tumor development was detected in orexin-A-treated mice. The effect of orexin-A in
Reducing tumor volume was also observed with other doses of peptide (Fig. 5A, inset). After a 15 day treatment with 0.112, 1.12 and 11.2 µmoles orexin-A/Kg, the tumor volumes were decreased by 62, 78 and 81 %, respectively. In another set of experiments, treatment of LoVo tumors in nude mice was initiated 7 days after inoculation of LoVo cells. It appeared that orexin-A (1.12 µmoles/Kg) very rapidly and strongly reversed the development of established tumors (Fig. 5B). Immunohistological examination of LoVo tumors developed in control and orexin-A-treated mice revealed that they were poorly differentiated adenocarcinomas in both cases (not shown). Characterization of apoptosis in LoVo tumors developed in nude mice was followed by immunohistochemical staining of activated caspase-3 in paraffin sections of tumors (Supplementary Fig. 4). Weak and intense stainings of activated caspase-3 were observed in control and orexin-A-treated mice, respectively. Quite interestingly, the amount of OX1R mRNA measured by RT-qPCR in resected LoVo tumors was very similar in control mice and orexin-A-treated mice (not shown), suggesting that long-term treatment with orexin did not downregulate OX1R mRNA levels.

Two other human colon cancer cell lines which expressed or not OX1R (Fig. 3), were also xenografted in nude mice e.g. HT29 and HCT116, respectively. Treatment of mice developing HT29 tumors with orexin-A resulted in drastic reduction of tumor volume (Fig. 5C) whereas no effect could be observed with HCT116 tumors (Fig. 5D).
Discussion

In this work, we showed that ectopic expression of the orexin receptor OX1R is a very frequent event in colon tumorigenesis regardless of the location of primary cancer in proximal or distal large intestine and also of their severity from Duke’s A to Duke’s C grade. Indeed, OX1R is expressed in all 38 colorectal tumors tested but not in normal glands, still normal colonocytes and control non tumoral colonic mucosa of patients with irritable bowel syndrome. This aberrant expression is maintained after metastatic migration of colon cancer cells to the liver which does not itself express OX1R in healthy conditions. We also showed that activation of OX1R by exogenous orexins in colon cancer cell lines established from primary tumors or metastases results in robust cell apoptosis and subsequent reduction in cell number. The proapoptotic action of orexins is also instrumental in colon cancer cells which have been selected (21) for their ability to resist to the most commonly used drug in colon cancer chemotherapy i.e. 5-fluorouracil (4, 27). Finally, orexins also strongly decrease the development of tumors in vivo in two models of colon cancer cells xenografted in nude mice. Altogether our data suggested that the orexin receptor OX1R represents a new promising gate to apoptosis in colon cancers which could be openable by exogenous orexins or OX1R agonists.

The molecular mechanisms whereby OX1R is aberrantly expressed during colon cancer progression are still unknown. The gene encoding OX1R maps to the human chromosomal region 1p33 (7). Chromosome 1p and the 1p32-36 loci have been shown to undergo genetic changes including deletions in human colorectal tumorigenesis (28). It could be hypothesized that deletions in chromosome 1 may hinder the repression of OX1R gene that occurs in normal colon.
OX1Rs are expressed in all hepatic metastases of colorectal cancer (Figure 2) and in colon cancer cell lines established from lymph nodes, ascite or lung metastases (Figure 3A). This is an important point for two reasons: i) the status of a given receptor may be different in primary tumors and metastases. For example, the EGFR status in primary colorectal tumors does not correlate with EGFR expression in related metastatic sites (29). A loss of EGFR expression was observed in a significant number of lymph node and liver metastases (29, 30), an event which has evident implication for treatment with EGFR-targeted antibodies (29). The status of OX1R appears to be similar in primary colorectal tumors and liver metastases. This is crucial if we consider that OX1R could represent a new target in colon cancer therapy or for metastases imaging. Indeed, the possibility to use OX1R-targeted agonists in order to induce apoptosis of cancer cells is more relevant in metastases, including micrometastases, than in the primary tumor which can be resected; ii) the liver is the main site of colorectal cancer metastasis. The status of OX1R in hepatic metastases appears to be suitable for imaging of micrometastases. The high affinity of OX1R for orexins makes this target convenient for radioisotopic imaging with $^{111}$In-labeled OX1R agonists as described previously for in vivo imaging of tumors with radiolabeled peptides (31). For imaging, it is also crucial that normal liver cells do not express OX1R (Figure 2). In that respect, OX1R appears to be much more interesting for imaging than vasoactive intestinal peptide (VIP) receptors (32) which are not only expressed in colon cancers (33) but also highly expressed throughout the normal human gut (34, 35) and liver (36).

The current view of the 7-pass transmembrane OX1R mediating apoptosis follows a mechanism that involves orexin binding at the plasma membrane triggering tyrosine phosphorylation of two tyrosine-based motifs ITIM (15) and ITSM (16) in
OX1R and subsequent recruitment and activation of the tyrosine phosphatase SHP-2 (13, 15, 16). The first steps of OX1R-driven apoptosis appear to occur at the plasma membrane level in agreement with the localization of OX1R at the cell surface of tumors and cultured cells. However, OX1R was also detected intracellularly in cytoplasm and/or nucleus in colon tumors and HT29 cells. The reason why OX1R is expressed intracellularly in colon cancers and its biological significance, if any, are not known. Many G protein-coupled receptors have been shown to be resident at intracellular sites (37-39). The presence of intracellular GPCRs in colon cancer cells has been previously reported for other peptide receptors (40) and alteration of receptor trafficking in cancer cells has been reported (41).

Failure of tumor cells to undergo apoptosis translates into malignant potential and chemotherapeutic resistance (18). Any means to selectively induce apoptosis of cancer cells versus normal cells should be considered as of tremendous interest (42). The ability of orexins to promote apoptosis in colon cancer cells in culture is well correlated to the expression of OX1R mRNA supporting the idea that OX1R level is determinant for the induction of apoptosis. Since all colorectal tumors and hepatic metastases express OX1R, the tumor cells should be amenable to apoptosis in vivo if simulated by OX1R agonists. Most of the colon cancer cell lines established from primary tumors or metastases undergo apoptosis upon challenge with orexins. In contrast, normal colonic epithelial cells and liver cells do not express OX1R and do not undergo orexin-induced apoptosis thereby (6). This represents an important feature in view of the possible use of OX1R as a therapeutic target. OX1Rs induce apoptosis through the intrinsic or mitochondrial pathway (6). Colon cancer cells also express Fas receptors (43) which, as death receptors, induce apoptosis through the extrinsic apoptosis pathway (17, 18, 42). Unfortunately, most colon cancer cell lines
are resistant to Fas ligand-mediated apoptosis even if they are positive to FasR (18). In contrast, normal colonic cells and hepatocytes remain exquisitely sensitive to Fas-mediated apoptosis (18) strongly limiting the potential use of FasR agonists as candidates for chemotherapeutic intervention (18). Attempts to use TNF and Fas ligand as an apoptosis-based therapy in colon cancer have also been thwarted by induction of NFκB-mediated inflammation and fulminant hepatic failure, respectively (42). The OX1R clearly does not suffer from such limits since orexins do decrease tumor development in vivo in models of human colon cancer cells xenografted in nude mice and do not bring about any observable adverse effect upon long-term treatment whatever the doses of orexin-A used up to 11.2 µmoles/kg.

In vivo experiments with human colon cancer cells xenografted in nude mice show that orexin-A is very efficient in slowing the tumor growth. It works at dose as low as 0.112 µmoles/kg and reaches a maximal effect at 1.12 µmoles/Kg. Not only orexin-A is able to drastically slow tumor growth when administered at the same time as cell inoculation (day 0), but most interestingly it also reverses very rapidly the growth of already established tumors (see Fig. 5B). It has been shown previously that orexins inhibit cultured colon cancer cell growth by inducing apoptosis without altering cell cycle (6). It is likely that orexins also reduced tumor growth in vivo by promoting apoptosis since we showed by immunostaining (see supplementary Fig. 4) that caspase-3 is activated in tumors upon orexin treatment.

The orexin receptor OX1R in colon cancer might be considered as a new type of gene in cancer since it is aberrantly expressed as a functional protein whose function, when activated by an agonist, is to promote apoptosis of the cancer cell. The colon cancer cells unexpectedly provide a new gate to promote their death by apoptosis which was not present in the normal colonocyte from which they derive. In
In this context, an important question arises: are OX1Rs activated in colorectal primary tumors or metastases \textit{in vivo}? Several lines of evidence support that the answer is no: i) colon tumors do not express the orexin precursor mRNA, suggesting that OX1Rs in colon cancer cells might not be activated by an autocrine loop; ii) the major source of orexins is the hypothalamus. The sites of synthesis of orexins in the periphery are still debatable (11, 13). Though early immunohistochemical studies detected orexins in small intestine, stomach and pancreas in rodents (26), further RT-qPCR experiments identified orexin precursor mRNA in rat testis but not in most other peripheral tissues including gut and liver (12). In the present paper, we demonstrated that normal human colonic mucosa and liver tissues do not express the orexin precursor mRNA; iii) a few studies reported the radioimmunological detection of very low concentrations of orexin-A in human plasma but results between laboratories probably need to be interpreted with caution (11). Anyway, the reported levels of orexin-A in human plasma are very low, in the range between 2 and 40 pM, in comparison of the Kd of the human OX1R i.e. 7 nM (16), making very unlikely that the elusive blood-borne orexins can activate OX1R in colon cancer. Altogether, our data and the literature suggest that the OX1Rs aberrantly expressed in colon cancer cells are most probably not activated by endogenous orexins \textit{in vivo}. Therefore, it may be suggested that OX1Rs in colorectal cancer constitute a gate to apoptosis which probably remains unopened \textit{in vivo} but could be openable by therapeutic administration of exogenous orexins or OX1R agonists. The development of long-lived peptide agonists or non-peptide agonists of orexin receptors will represent an important advance not only in neuroscience (44) but also in colon cancer research. The OX1R, orexins and forthcoming OX1R agonists might be novel candidates for colorectal cancer therapy.
In conclusion, we provide evidence that OX1R is aberrantly expressed in all primary colorectal tumors and metastases and that its activation by exogenous orexins result in robust apoptosis of colon cancer cells in culture and strong decrease of tumor development in mice xenografted with colon cancer cells *in vivo*. Another remarkable property of OX1R-mediated apoptosis in colon cancer cells is that it remains efficient in HT29-FU cells that have been selected for their ability to resist to 5-fluorouracil. In this context, the orexin receptor OX1R represents an Achilles heel of colon cancer and is a new promising therapeutic target.
Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

We thank Thécla Lesuffleur from UMR-S 938 INSERM (France) for her generous gift of the HT29-FU cell line.

Grant Support

Aadil El Firar is supported by Ministère de la Recherche et de l’Enseignement Supérieur, and Fondation pour la Recherche Médicale (FRM).
References


LEGENDS TO FIGURES

**Figure 1.** Indirect immunostaining of OX1R in human colon tumors. (A) Paraformaldehyde-fixed sigmoid from a patient with irritable bowel. No immunoreactive signal was observed. (B-G) Paraformaldehyde-fixed colon tumors. (B) Strong immunostaining in neoplastic glands whereas normal glands remained negative. (C) Detail of this tumor showing the difference of immunostaining in the same glands between neoplastic cells and still normal colonocytes. (D) Invasive cancer of the sigmoid. (E) Same tumor : negative control after incubation of the OX1R antibody with its homologous immunogen peptide. (F) Invasive cancer of the right colon (G) Same tumor, the signal was negative after incubation of the OX1R antibody with its homologous immunogen peptide. Bar = 200 µm for (B), 50 µm for (A, C, D, E) and 20 µm for (F, G).

**Figure 2.** Expression of OX1R mRNA and OX1R immunostaining in liver metastasis from colorectal tumors. (A) Relative OX1R mRNA expression in 10 metastases and 7 adjacent liver tissues was determined by RT-qPCR and calculated as described in Methods. For metastases, individual values are shown in a scatter plot and the mean value is 51 “arbitrary unit”. (B) Indirect immunostaining of OX1R in paraformaldehyde-fixed liver metastasis of a colonic carcinoma. (C) Negative control obtained by omitting the primary antibody. Bar = 20 µm.
Figure 3. Expression of OX1R mRNA and effect of orexine-A on cell apoptosis in human colon cancer cell lines. (A) Relative OX1R mRNA expression in cell lines was determined by RT-qPCR and was calculated as described in Methods. (B) The proapoptotic effect of orexin-A in cells was investigated as described in Materials and Methods. Results are expressed as means ± SE (3 determinations). *** P < 0.001 and ns, non significant vs untreated cells. (C) Correlation between the proapoptotic response to orexin challenge and the expression of OX1R mRNA in colon cancer cell lines as determined in (A). r = 0.86.

Figure 4. Effect of orexin on apoptosis and cell number in HT29-FU cells in the presence or absence of 5-fluorouracil. Cells, grown in the presence of 10% FCS, were treated with (black bars) or without (open bar) orexin-A for 48 h in the presence of 5-FU at the indicated concentrations. (A) proapoptotic effect of orexin-A (1 µM) in parental HT29-FU cells. Results are expressed as the percentage of orexine-A induced apoptotic cells. Background apoptosis without 5-FU and orexine-A was 4.4 ± 0.3 % and was subtracted from the values shown in the figure. (B) antigrowth effect of orexin-A (1 µM) in HT29-FU (B) cells. Cells excluding trypan blue were counted, *** p < 0.001 vs untreated cells.

Figure 5. Effect of daily inoculation of orexin-A on the growth of tumors developed by xenografting human colon cancer cells in nude mice. LoVo cells (A and B), HT-29 cells (C) or HCT116 cells (D) were inoculated in the flank of nude mice at day 0. Mice were daily injected intraperitoneally with 100 µl of orexin-A solutions starting at day 0(○) or day 7 (triangle) or with 100 µl of PBS (●) for controls. The daily treatment corresponded to 1.12 µmoles of orexin-A/Kg (A, B, C and D), 0.112 or 11.2
μmoles/Kg (A, inset). The development of tumors was followed by caliper measurement. Data are means ± SE of 6 tumors in each group. * p < 0.01 versus control.
### Table 1. OX1R immunoreactivity in cancers of the proximal and distal colon

<table>
<thead>
<tr>
<th>Proximal colon Cases</th>
<th>Duke’s stage</th>
<th>Tumor site</th>
<th>Percent of stained cells</th>
<th>Staining intensity</th>
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<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>Cecum</td>
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<td>± to +++</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>Right</td>
<td>80</td>
<td>+ to ++</td>
</tr>
<tr>
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<tr>
<td>4</td>
<td>A</td>
<td>Right</td>
<td>90</td>
<td>± to +</td>
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<tr>
<td>5</td>
<td>B</td>
<td>Cecum</td>
<td>85</td>
<td>+ to ++</td>
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<td>B</td>
<td>Cecum</td>
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<td>±</td>
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<td>7</td>
<td>B</td>
<td>Cecum</td>
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<td>+ to ++</td>
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<tr>
<td>8</td>
<td>B</td>
<td>Cecum</td>
<td>70</td>
<td>± to +</td>
</tr>
<tr>
<td>9</td>
<td>B</td>
<td>Right</td>
<td>50</td>
<td>± to ++</td>
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<tr>
<td>10</td>
<td>B</td>
<td>Right</td>
<td>65</td>
<td>± to +</td>
</tr>
<tr>
<td>11</td>
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<td>12</td>
<td>B</td>
<td>Right</td>
<td>80</td>
<td>± to +++</td>
</tr>
<tr>
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<td>C</td>
<td>Cecum</td>
<td>90</td>
<td>+</td>
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<td>14</td>
<td>C</td>
<td>Cecum</td>
<td>60</td>
<td>±</td>
</tr>
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<td>15</td>
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<td>Cecum</td>
<td>90</td>
<td>+ to ++</td>
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<td>C</td>
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<td>± to +</td>
</tr>
<tr>
<td>17</td>
<td>C</td>
<td>Right</td>
<td>100</td>
<td>+ to ++</td>
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<tr>
<td>18</td>
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<td>+</td>
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<tr>
<td>20</td>
<td>C</td>
<td>Right</td>
<td>80</td>
<td>± to ++</td>
</tr>
<tr>
<td>21</td>
<td>C</td>
<td>Right</td>
<td>100</td>
<td>+ to +++</td>
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<table>
<thead>
<tr>
<th>Distal colon cases</th>
<th>Duke’s stage</th>
<th>Tumor site</th>
<th>Percent of stained cells</th>
<th>Staining intensity</th>
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<tbody>
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<td>A</td>
<td>Left</td>
<td>100</td>
<td>±</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>Left</td>
<td>70</td>
<td>± to +</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>Sigmoid</td>
<td>100</td>
<td>++ to +++</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>Sigmoid</td>
<td>90</td>
<td>+ to +++</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
<td>Rectosigmoid</td>
<td>50</td>
<td>±</td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td>Left</td>
<td>95</td>
<td>++</td>
</tr>
<tr>
<td>7</td>
<td>B</td>
<td>Left</td>
<td>85</td>
<td>+ to +++</td>
</tr>
<tr>
<td>8</td>
<td>B</td>
<td>Sigmoid</td>
<td>50</td>
<td>±</td>
</tr>
<tr>
<td>9</td>
<td>B</td>
<td>Sigmoid</td>
<td>80</td>
<td>±</td>
</tr>
<tr>
<td>10</td>
<td>B</td>
<td>Sigmoid</td>
<td>80</td>
<td>± to +</td>
</tr>
<tr>
<td>11</td>
<td>B</td>
<td>Rectum</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>C</td>
<td>Left</td>
<td>95</td>
<td>± to ++</td>
</tr>
<tr>
<td>13</td>
<td>C</td>
<td>Left</td>
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<td>+ to ++</td>
</tr>
<tr>
<td>14</td>
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<td>++</td>
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<td>15</td>
<td>C</td>
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<td>50</td>
<td>±</td>
</tr>
<tr>
<td>16</td>
<td>C</td>
<td>Sigmoid</td>
<td>70</td>
<td>± to +</td>
</tr>
<tr>
<td>17</td>
<td>C</td>
<td>Sigmoid</td>
<td>90</td>
<td>± to ++</td>
</tr>
</tbody>
</table>

Staining intensity was variable from place to place and scored as: ±, discrete to weak; +, moderate; ++, strong; ++++, intense.
Table 2: Relative OX1R, OX2R and preproorexin mRNA expression in various colon tumors tissues.

<table>
<thead>
<tr>
<th>Localization</th>
<th>Duke’s stage</th>
<th>OX1R</th>
<th>OX2R</th>
<th>preproorexin</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Left distal colon</td>
<td>A</td>
<td>58 ± 7</td>
<td>nd</td>
</tr>
<tr>
<td>T2</td>
<td>Left distal colon</td>
<td>B</td>
<td>69 ± 9</td>
<td>nd</td>
</tr>
<tr>
<td>T3</td>
<td>Left distal colon</td>
<td>C</td>
<td>43 ± 7</td>
<td>nd</td>
</tr>
<tr>
<td>T4</td>
<td>Left distal colon</td>
<td>C</td>
<td>58 ± 3</td>
<td>nd</td>
</tr>
<tr>
<td>T5</td>
<td>Left distal colon</td>
<td>D</td>
<td>143 ± 16</td>
<td>nd</td>
</tr>
<tr>
<td>T6</td>
<td>Left distal colon</td>
<td>D</td>
<td>32 ± 6</td>
<td>nd</td>
</tr>
<tr>
<td>T7</td>
<td>Sigmoïd distal colon</td>
<td>A</td>
<td>137 ± 18</td>
<td>nd</td>
</tr>
<tr>
<td>T8</td>
<td>Sigmoïd distal colon</td>
<td>C</td>
<td>187 ± 23</td>
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<tr>
<td>T9</td>
<td>Right proximal colon</td>
<td>A</td>
<td>48 ± 9</td>
<td>nd</td>
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<tr>
<td>T10</td>
<td>Right proximal colon</td>
<td>B</td>
<td>70 ± 11</td>
<td>nd</td>
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<td>nd</td>
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<td>nd</td>
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<td>nd</td>
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<td>NTL</td>
<td>Liver</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>Control tissue</td>
<td>Hypothalamus</td>
<td>169 ± 12</td>
<td>75 ± 6</td>
<td>190 ± 21</td>
</tr>
</tbody>
</table>

T1 to T10 represent proximal and distal colonic tumors from 10 different patients. NTC1 to NTC5 represent non tumoral proximal and distal colonic mucosae from 5 different patients with irritable bowel syndrome. NTL is a representative non tumoral liver sample adjacent to a colorectal metastasis. All RNAs were prepared from frozen collected tissues. Human hypothalamus cDNAs were used as positive controls. Results are expressed as means ± SE (4 determinations); nd, not detected.
Figure 2

A

Relative OXIR mRNA expression (OXIR/GADPH)

0.1

Adjacent normal liver

Liver metastasis

B

C

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Figure 4

A

% of oocytes induced apoptosis

B

Cell number (x10^4)

0 1 10

0

5-FU (μM)

0 1 10

0

5-FU (μM)
Figure 5
Aberrant expression of OX1 receptors for orexins in colon cancers and liver metastases: an openable gate to apoptosis

Thierry Voisin, Aaïl El Firar, Magali Fasseu, et al.

Cancer Res Published OnlineFirst March 17, 2011.

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doi: 10.1158/0008-5472.CAN-10-3473

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