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Up-regulation of FXR isoforms is not required for stimulation of the expression of genes involved in the lack of response of colon cancer to chemotherapy

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ABSTRACT

Several mechanisms are involved in the poor response of colorectal adenocarcinoma (CRAC) to pharmacological treatment. Since preliminary evidences have suggested that the enhanced expression of farnesoid X receptor (FXR) results in the stimulation of chemoresistance, we investigated whether FXR up-regulation is required for the expression of genes that characterize the multidrug resistance (MDR) phenotype of CRAC. Samples of tumours and adjacent healthy tissues were collected from naive patients. Using Taqman Low-Density Arrays, the abundance of mRNA of 87 genes involved in MDR was determined. Relevant changes were re-evaluated by conventional RT-QPCR. In healthy tissue the major FXR isoforms were FXRα2(+/−) (80%). In tumours this predominance persisted (91%) but was accompanied by a consistent reduction (3-fold) in total FXR mRNA. A lower FXR expression was confirmed by immunostaining, in spite of which there was a significant change in the expression of MDR genes. Pharmacological challenge was simulated "in vitro" using human CRAC cells (LS174T cells). Short-term (72 h) treatment with cisplatin slightly increased the almost negligible expression of FXR in wild-type LS174T cells, whereas long-term (months) treatment induced a cisplatin-resistant phenotype (LS174T/R cells), which was accompanied by a 350-fold up-regulation of FXR, mainly FXRα1(+/−). However, the changed expression of MDR genes in LS174T/R cells was not markedly affected by incubation with the FXR antagonist Z-guggulsterone. In conclusion, although the enhanced expression of FXR may be involved in the stimulation of chemoresistance that occurs during pharmacological treatment, FXR up-regulation is not required for the presence of the MDR phenotype characteristic of CRAC.

1. Introduction

When data regarding both sexes are considered together, colorectal adenocarcinoma (CRAC) is the third most common cancer in Western countries, after lung cancer (more frequent in men) and breast cancer (more frequent in women). Moreover, CRAC is the second leading cause of death due to cancer [1]. Recent advances have permitted improvements in early diagnosis, which is particularly important in this case because surgery can be curative in more than 90% of patients with CRAC if they are diagnosed during the early stages [2]. The treatment of choice for CRAC is resection of the affected segment. Nevertheless, neoadjuvant radiochemotherapy is often useful for reducing local-regional recurrences and chemotherapy improves the prognosis of patients with advanced CRAC [2], which has contributed to the fact that, in recent years, the median survival time in patients with metastases due to CRAC has increased from 5 to 24 months [3]. However, although the rate of success of pharmacological regimens in the treatment of CRAC is higher than in other gastrointestinal tumours, cancer cells often develop resistance during the pharmacological treatment through up-regulation of the genes involved in a variety of mechanisms of chemoresistance (MOCs), whereas on many other occasions chemoresistance is present even before treatment has begun [4].

Abbreviations: CDDP, *cis*-diamminedichloroplatinum(II); CRAC, colorectal adenocarcinoma; 5-FU, 5-fluorouracil; FXR, farnesoid X receptor; MDR, multidrug resistance; MOC, mechanism of chemoresistance; TKI, tyrosine kinase inhibitor; TLDA, Taqman Low-Density Array.

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Although the farnesoid X receptor (FXR) has been considered a specific bile acid receptor involved in the control of the expression of genes accounting for lipid homeostasis [5], we have recently reported preliminary data indicating that the activation of FXR can be triggered, in a bile acid-independent manner, by the exposure of liver cells to certain toxic compounds, such as cisplatin (*cis*-diamminedichloroplatinum(II), CDDP) [6]. These findings have suggested the question as to whether an enhanced expression of this nuclear receptor might be involved in the development of the multidrug resistance (MDR) phenotype that is characteristic of CRAC.

In humans, FXR is encoded by the *NR1H4* gene, whose expression can result in four isoforms with relative tissue/cell-specific distributions [7]. To date, these isoforms have been classified using different nomenclatures. Here we have designated them as FXRα1(+), FXRα1(−), FXRα2(+) and FXRα2(−), based on their difference in the initial region (exons 1–3) of mRNA (α1 and α2) and the presence (+) or absence (−) of a 12 base-pair insert at the end of exon 5.

Both under physiological circumstances and regarding their role in inducing chemoresistance in tumours, the functional differences of FXR isoforms are poorly understood. Thus, the aim of the present study was to determine whether the overall expression of FXR or any of its isoforms are modified in CRAC and whether this is related to the expression of genes involved in the MDR phenotype of these tumours. In the present study, in order to characterize the genetic profile of genes involved in MDR phenotype individually, Taqman Low Density Arrays (TLDAs) have been used. The genes included in the microfluidic cards have been previously reported to play a role in MOC by: affecting drug uptake (MOC-1a) or efflux (MOC-1b), changing intracellular metabolism (pro-drug activation or drug inactivation) (MOC-2), changing the expression/functionality of molecular targets (MOC-3), enhancing DNA repair machinery (MOC-4), reducing activation of apoptosis (MOC-5a) or enhancing expression/activity of anti-apoptotic pathways (MOC-5b) [4].

2. Methods

2.1. Human samples

Tumour samples from CRAC were obtained for therapeutic/diagnostic/prognostic purposes from surgically removed tumours or biopsies. At the time of sample collection the patients had not yet received any antitumour treatment. The research protocol, which complied with the ethical guidelines of the 1975 Declaration of Helsinki, was reviewed and approved by the Human Subjects Committee of the University of Salamanca, and written consent was obtained in all cases. Fresh tissue specimens collected in the operating room were immediately immersed in RNAlater solution (Ambion, Life Technologies, Madrid, Spain). Approximately the same proportion of males and females was included in all groups.

2.2. Cell cultures

LS174T human colon adenocarcinoma cells were from the American Type Culture Collection (Manassas, VA). A monoclonal subline partly resistant to CDDP had been previously obtained at our laboratory [8]. The cells were cultured with appropriate medium in a humidified CO_2:air (5:95%) atmosphere at 37 °C. To calculate the IC50 for CDDP, approximately 5×10^3 or 5×10^4 cells/well (depending on the cell line) were seeded in 96-well plates. To determine cell viability in order to calculate the IC50 from the dose–response curves, the formation of formazan from the tetrazolium salt (Thiazolyl Blue Tetrazolium Bromide, MTT, from Sigma–Aldrich Quimica, Madrid, Spain) by living cells was used after long-term (72 h) exposure to CDDP. Cells incubated with or without CDDP at concentrations close to the IC50 (LS174T: 20 μM and LS174T/R: 100 μM) for 72 h were analysed using TLDAs to characterize MDR expression profiles. Effect of incubation of LS174T/R cells with the FXR antagonist Z-guggulsterone (Santa Cruz Biotechnology) (10 μM for 48 h) [9] on the expression of MDR genes and some typical FXR target genes was also investigated. Experiments were carried out in triplicate in at least three different cultures per cell line.

2.3. Measurement of mRNA levels

Total RNA extraction from human CRAC samples and cultured cells was performed as previously described [10]. Retrotranscription was carried out using the "SuperScript® VILO™ cDNA Synthesis Kit" (Invitrogen, Barcelona, Spain). QPCR, using Amplitaq Gold polymerase, was performed in an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Madrid, Spain) for single reactions to determine mRNA by conventional RT-QPCR with SYBR® Green I detection, or using TLDAs in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) with TaqMan Mix detection to analyse the expression of genes involved in MOCs (Supplementary Table 1). In all cases, the following thermal conditions were used: a single cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The primer oligonucleotides for FXR and its isoforms (Supplementary Table 2) and for the MOC genes, together with their appropriate Taqman probes to be included in the microfluidic cards, were designed and validated by us (FXR) or by Applied Biosystems (TLDAs) (data not shown). As house-keeping genes, 18S rRNA, *ACTB* and *GAPDH* were included in the TLDAs. 18S rRNA was used as a quality-control check among samples and groups. Since the Ct values for *ACTB* and *GAPDH* were closer to those of the target genes double normalization of the results in each sample was carried out with the values obtained for these two genes. Expression levels were calculated as $2^{-\Delta\Delta Ct}$, where ΔCt is the difference of Ct in each sample between the target gene and the normalizer. This was used to calculate ΔΔCt as the difference in this value between tumour tissue and paired adjacent healthy colon tissue. To carry out the absolute determination of the number of mRNA copies of FXR isoforms and GAPDH, each cDNA was obtained by high-fidelity RT, cloned into a pGEMT Easy vector (Promega, Madrid, Spain), and sequenced. This construct was amplified in *Escherichia coli* and used as a template to obtain by PCR, followed by gel electrophoresis, different amounts of a purified fragment to build the standard curves required for the absolute determination of mRNA by QPCR.

2.4. Immunohistochemistry

Using the "Immunohistochemistry Accesory Kit" (Bethyl Laboratories, Inc., Montgomery, TX), FXR immunostaining was performed on tissue microarrays (TMAs) obtained from the Biobanc of Barcelona Clinic Hospital-IDIBAPS (Barcelona, Spain). Formalin-fixed paraffin-embedded TMAs contained 15 human CRAC and paired non-neoplastic tissue from resection margins. After deparaffinization in xylene and rehydrated in a graded series of ethanol, the endogenous peroxidase activity was quenched with freshly prepared 0.3% H_2O_2 in methanol for 30 min at room temperature, and samples were subjected to antigen retrieval using steamer heating for 20 min. Tissue sections were incubated for 1 h with the rabbit polyclonal antibody (H-130, Santa Cruz Biotechnology) diluted 1:100. Subsequently, the slides were rinsed 3× in PBS, incubated with peroxidase-conjugated anti-rabbit antibody for 1 h, again rinsed 3× in PBS and incubated with the peroxidase