DHA induces apoptosis and differentiation in human melanoma cells in vitro: involvement of HuR-mediated COX-2 mRNA stabilization and β-catenin nuclear translocation

Simona Serini1, Elena Fasano1, Elisabetta Piccioni, Giovanni Monego1, Achille R.M. Cittadini, Leonardo Celleno2,3, Franco O. Ranelletti4 and Gabriella Calviello1

Institute of General Pathology, 1Institute of Human Anatomy, 2Institute of Dermatology, 3Cosmetological Research Centre and 4Institute of Histology, Università Cattolica del Sacro Cuore, Largo F. Vito, 1 - 00168 Rome, Italy
Fax: +39-06-3386446; Email: g.calviello@rm.unical.it

The pro-inflammatory phenotype accompanying melanoma progression includes an enhanced expression of cyclooxygenase-2 (COX-2), which plays an important role in the acquisition of apoptosis resistance, and is a suitable target for melanoma prevention and therapy. We observed that the WM266-4 metastatic melanoma cell line showed a constitutive COX-2 expression higher than that of the primary WM115 cells, an increased cytosolic level of the COX-2 messenger RNA (mRNA)-stabilizer human antigen R (HuR) and a lower susceptibility to basal apoptosis. The transfection of HuR siRNA induced apoptosis and reduced COX-2 protein abundance in both the cells. The same effects were observed treating the cells with the n-3 polyunsaturated fatty acid docosahexaenoic acid (DHA), which reduced the cytoplasmic location and expression of HuR and, correspondently, decreased COX-2 protein expression and induced apoptosis. DHA also decreased the expression and stability of COX-2 mRNA, increased the β-catenin expression in the nuclei and reduced it in the cytosol, where it forms a complex with HuR and COX-2 mRNA. DHA had also a pro-differentiating effect, which is compatible with the nuclear translocation of β-catenin. These findings allow us to associate for the first time the constitutive expression of COX-2 in melanoma cells to the HuR-mediated stabilization of its mRNA and suggest that also β-catenin may play a role in HuR-mediated COX-2 stabilization in these cells. The data demonstrate that the HuR-mediated stabilization of COX-2 may represent a target of DHA action in melanoma cells and suggest the application of DHA in the prevention and therapy of melanoma.

Introduction

Malignant melanoma is the most aggressive form of skin cancer, showing high tendency to metastasize. According to recent statistics, during the past few decades, there has been a considerable increase in its incidence worldwide (1). Several risk factors have been identified, including exposure to sunlight, naevus count, phototype, family history of melanoma and, hypothetically, exposure to artificial light (2). Evidence has been found that inflammation also exerts a decisive role in the development and progression of melanoma (3). It was observed that the activation of nuclear factor-kappaB signaling, considered a hallmark of inflammation, induced proliferation and apoptosis resistance in melanoma cells in vitro and was required for the development and progression of tumors arising in a transgenic mouse melanoma model (3). Moreover, it was reported that metastatic melanoma cells typically express a pro-inflammatory phenotype (4). This is revealed by the endogenous expression of inflammatory cytokines and their receptors as well as the constitutive overexpression of nuclear factor-kappaB- and STAT3-dependent genes, including that encoding cyclo-oxygenase-2 (COX-2) (4). This enzyme is known to play a critical role in the inflammatory response, and COX-2 inhibitors have been largely used as inflammation suppressors (5). Moreover, the prostanooids originating from arachidonic acid through COX-2 catalysis are known to enhance proliferation of tumor cells, protect them against apoptosis and induce tumor neo-angiogenesis and invasion (6,7). The constitutive expression of COX-2 has been associated with the development and progression of many kinds of cancers (8), including melanoma (7,9) and has been related to apoptosis resistance (10), a primary cause of treatment failure in this cancer. For these reasons, COX-2 expression has been suggested as a biomarker to distinguish early melanomas from benign melanocytic lesions (11) and establish the prognosis in melanoma patients. It was also proposed as a suitable target for therapy and prevention in melanoma (12) and various other cancers (13). The carcinogenic role of COX-2 in melanoma is also supported by the anti-neoplastic effects of COX-2 inhibitors in melanoma cell lines (14,15) and by the favorable course reported for melanoma patients treated with COX-2 inhibitors (12,16). However, the use of these drugs has been hampered by their severe cardiovascular and gastrointestinal side effects (17). Components of our diet, such as n-3 polyunsaturated fatty acids (PUFAs), are known to powerfully inhibit the enhanced expression and increased activity of COX-2 in tumors (18–21). For almost 20 years now, we have been studying the anti-neoplastic activity of n-3 PUFAs (22–24). We found that the pro-apoptotic and anti-angiogenic effects exerted by n-3 PUFAs in colon cancer cells were strictly related to their ability to inhibit COX-2 expression and PGE2 production (20,21). Docosahexaenoic acid (DHA), one of the major dietary n-3 PUFAs, has been previously reported to induce apoptosis and cell cycle arrest in melanoma cells (25), and an additive effect was shown when combinations of DHA and the COX inhibitors indomethacin and celecoxib were used to reduce the growth of melanoma A375 cells (26). In agreement, it was also found (27) that n-3 PUFAs were able to inhibit the invasive potential of the highly metastasizing human melanoma cell line 70W by downregulating COX-2 messenger RNA (mRNA) and protein expression. We have recently observed (28) that DHA reverted the resistance to ultraviolet radiation-induced apoptosis arisen in human keratinocytes and related this effect to the DHA-induced reduction of COX-2 expression and to the decreased cytosolic expression of human antigen R (HuR), an ELAV-like protein that has been reported to powerfully stabilize COX-2 mRNA in keratinocytes subjected to UVB irradiation (29). The increased cytosolic location of HuR has been associated with high levels of COX-2 expression and high tumor stage in several kinds of cancer, including colon, breast, ovarian and gastric cancer (30–33). Besides COX-2 mRNA, HuR has been reported to stabilize mRNAs encoding other anti-apoptotic proteins, such as prothymosin, SIRT1, Bcl-2 and Mcl-1 (34). Since apoptosis resistance is a primary cause of treatment failure in melanoma and increases during melanoma progression (34), in this study, we investigated whether DHA could overcome the HuR-dependent stabilization of COX-2 mRNA and the anti-apoptotic action of HuR in human melanoma cell lines. For that, we took advantage of two naturally occurring primary and metastatic cell lines, WM115 and WM266-4, originally derived from the same patient.

Moreover, since it was previously shown in colon cancer cells and in several normal cells (mouse NIH3T3 cells and human embryonic kidney 293T cells) that β-catenin may bind to HuR and COX-2 mRNA to

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facilitate the stabilization of COX-2 mRNA (35), we explored whether a relationship could exist between β-catenin, HuR and COX-2 mRNA in melanoma cells and whether DHA could modulate the expression of β-catenin in these cancer cells.

Materials and methods

Cell lines

The primary WM115 and the metastatic WM266-4 melanoma cell lines were kindly gifted by Dr M.C. Failla (Istituto Dermopatico dell’Immacolata, Rome, Italy). Cells were grown in Eagle’s minimum essential medium containing 2 mM glutamine, non-essential aminoacids and Na-pyruvate. Human-immunolized HaCaT keratinocytes (which were previously demonstrated to express high levels of COX-2 mRNA (26) and, for this reason, utilized as a positive control in reverse transcription-polymerase chain reaction (RT-PCR) for COX-2 mRNA) were purchased from ATCC (Rockville, MD, USA) and grown in Dulbecco’s modified Eagle’s medium containing 2 mM glutamine and anti-biotics (100 U/ml penicillin and 100 μg/ml streptomycin). All cells were supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere containing 5% CO2. Cell lines were serially subcultured by treatment with trypsin-ethylenediaminetetraacetic acid (EDTA) and seeded twice a week at a density of 3 x 10^5 cells/ml. DHA, eicosapentanoic acid (EPA, 20:4 n-3), linoleic acid (18:2 n-6) and oleic acid (18:1 n-9) were purchased from Sigma–Aldrich (Sigma, St Louis, MO, USA).

Fatty acids were added from an absolute ethanol stock solution, and the control cells were treated with the same amount of vehicle alone. The final ethanol concentration never exceeded 0.5% (vol/vol).

Cell growth evaluation

WM115 and WM266-4 melanoma cells were seeded in 24-well culture plates at a density of 6 x 10^4 cells per well and exposed to increasing DHA concentrations (1–30 μM) for 48 h. Cells were then harvested and exposed to the impermeant dye trypan blue (0.1% wt/vol for 5 min). Cells were counted under an inverted-phase microscope using a Burker chamber (quadruplicate hemocytometer counts of triplicate cultures were performed). Only viable cells, excluding the dye, were comprised in the count of viable cells. IC50 (the dose of DHA which induced a 50% growth inhibition) was calculated for each cell line utilizing GraphPad Prism 5 software.

Apoptosis detection

The percentage of apoptotic cells was evaluated by acridine orange–ethidium bromide method (36) and analyzed by fluorescence microscopy.

Apoptosis was also assessed by fluorometrically measuring the activity of caspase-3. Briefly, cells were incubated for the indicated times and then harvested. Cells (2 x 10^6) were lysed in 100 μl of 50 mM Tris–HCl buffer, pH 7.5, containing 0.5% NP-40, protease, and phosphatase inhibitors. After incubation for 10 min at 4°C and centrifugation for 10 min at 12 000g to remove cell debris, cell lysates were collected and subjected to Western blot analysis. Whole cell extracts were prepared by lysing the cells (1 x 10^6) in ice-cold lysis buffer (1 mM MgCl2, 350 mM NaCl, 20 mM HEPES, 0.5% NP-40, 0.1 mM EDTA, 1 mM Na3PO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1.5 mM leupeptin, 20% glycerol, 1% NP-40) for 30 min at 4°C and centrifuging for 10 min at 12 000g to remove cell debris. To obtain cytosolic protein fractions, cells were lysed in protein lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.625% NP-40, protease and phosphatase inhibitors]. After incubation at 4°C for 10 min, nuclei were pelleted by centrifugation at 5000 r.p.m. for 5 min and the supernatant was kept as the cytoplasmic fraction (which was further centrifuged for three times to remove remaining particulates). Nuclei were washed twice by centrifugation (1500g for 10 min at 4°C) in a buffer containing 10 mM HEPES (pH 7.5), 10 mM KCl, 1 mM DTT, 1 mM MgCl2 and resuspended in nuclear lysis buffer [20 mM HEPES (pH 7.5), 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2; 25% glycerol] containing protease inhibitors on ice for 15 min with frequent vortexing. Lysed nuclei were centrifuged at 15 000g for 5 min at 4°C, and supernatant was saved as the nuclear fraction. The protein content of whole cell lysates, cytosolic fraction and nuclear fraction lysates was determined by Bradford method using the Biorad assay (Hercules, CA, USA).

Equal amounts of proteins (50 μg) were separated on a 10% sodium dodecyl sulfate polyacrylamide gel and electroblotted on a nitrocellulose membrane. The membrane was blocked overnight at 4°C in 5% dried milk (wt/vol) in phosphate-buffered saline (PBS) plus 0.05% Tween 20 and then incubated with specific antibodies to COX-2 (clone 29, catalog # sc-19999), Bcl-2 (clone C-2, catalog # sc-7382), Bax (clone P-19, catalog # sc-526), HuR (clone 3A2, catalog # sc-5261) and β-catenin (clone H-102, sc-7199) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). As loading controls, the blots were probed with an anti-α-actin antibody (clone B-12, catalog # sc-66524; Santa Cruz Biotechnology) at 1:10 000 dilution, an anti-β-actin antibody (clone C-2, catalog # sc-8432; Santa Cruz Biotechnology) at a 1:200 dilution and an anti-SNRNP70 antibody (ab51266; Abcam, Cambridge, UK) at a 1:200 dilution. Following incubation with secondary mouse (COX-2, Bcl-2, HuR, α-actin, β-actin) or rabbit (Bax, β-catenin; SNRP70) antibodies (Amersham, Pharmacia Biotech Italia, Milan, Italy), the immunocomplexes were visualized using the enhanced chemiluminescence detection system (Amersham) and quantitated by densitometric analysis.

Extraction and analysis of COX-2 mRNA

Total RNA was extracted from cells using PureLink™ RNA mini Kit according to manufacturer’s protocols (Ambion by Life Technologies, Carlsbad, CA, USA). The RNA was eluted in diethylpyrocarbonate-treated water (0.01% diethylpyrocarbonate) and stored at −80°C until RT–PCR analysis. Nucleic acid concentrations were measured by spectrophotometry (Hewlett–Packard HP UV/Vis spectrophotometer 8450).

RT–PCR assay was performed using the two-step method. For the first-step of reverse transcription, we used QuantiTect Reverse® Transcription kit (Qiagen, Hilden, Germany) with 500 ng of total RNA as template RNA, following the manufacturer’s procedure.

For the second step of PCRs, we employed QuantiTect SYBR® Green Kits (Qiagen) and QuantiTect® Primer Assays (Qiagen) for human COX-2 and β-actin, according to manufacturer’s protocol described for the real-time thermal cycle LightCycler (Roche, Mannheim, Germany). PCR data obtained by the LightCycler software were automatically analyzed by the Relative Quantitation Software (Roche) and expressed as target/reference ratio. Our approach was based on the calibrator-normalized relative quantification including correction for PCR efficiency.

Evaluation of COX-2 mRNA stability

COX-2 mRNA stability was evaluated in both WM115 and WM266-4 cells pretreated or not with 30 μM DHA for 4 h by subsequently adding Actinomycin D (Act D, 10 μg/ml) to block transcription for an additional 60 min. At different time points (15, 30, 45 and 60 min), cells were harvested and the remaining COX-2 mRNA was analyzed by RT–PCR as described above.

Small interfering RNA transfection for knockdown of HuR

Small interfering RNA (siRNA) duplex oligonucleotides were purchased from Qiagen (Milan, Italy). The transfection was performed as indicated by the manufacturer. Briefly, WM266-4 cells were seeded at a density of 3 x 10^4 per plate in 100 mm plates for western blot analysis and at 3 x 10^5 per well in six-well plates for apoptosis detection. On the same day, cells were transfected with the siRNA-HuR-specific siRNAs or with a fluorescently labeled siRNA as a transfection efficiency control using the HiPerfect Transfection reagent (Qiagen). The concentration of siRNA in the cell culture medium was 10 nM. The target sequences were 5'-AAGTGGGCGGACAGCGTTG-3' (Hs-ELAVL1_1 siRNA) and 5’-ACCCGTTTCAATGGTGCTAATA-3’ (Hs-ELAVL1_11 siRNA). After 12 h, the efficiency of transfection was verified by fluorescence microscopy. After 72 h from transfection (maximum HuR silencing time), cells were evaluated for the expression of COX-2 and apoptosis induction. The two siRNAs used achieved the same extent of reduction in HuR expression, so the first one (Hs-ELAVL1_1) was used in the reported experiments. The effect of siRNA knockdown on cell viability was determined by trypan blue exclusion method.

Immunocytochemical analysis of intracellular HuR and β-catenin localization

WM266-4 cells were seeded in Nunc multi-well chamber slides at the density of 1 x 10^5 cells per well. After treatment, cells were fixed with 4% paraformaldehyde for 8 min, then permeabilized with 0.2% bovine serum albumin and 0.1% Triton X-100 in PBS for 8 min and washed with PBS. Subsequently, cells were labeled with HuR mouse monoclonal (clone 3A2, sc-526; Santa Cruz Biotechnology) or β-catenin rabbit polyclonal (clone H-102, sc-7199; Santa Cruz Biotechnology) antibodies diluted 1:500 in Normal Antibody Diluent (phosphate buffered). Following incubation, cells were washed several times with PBS. Binding was visualized using biotinylated secondary antibody and the streptavidin–biotin peroxidase complex developed with diaminobenzidine.

Immunoprecipitation of COX-2 mRNA–β-catenin–HuR complexes

The immunoprecipitation of COX-2 mRNA–β-catenin–HuR complexes was performed according to Peritz et al. (37) with some modifications. Briefly, WM266-4 cells (three 100 mm Petri culture dishes, containing 5 x 10^5 cells each) were harvested and lysates prepared by adding to each Petri dish 1 ml of
polysome lysis buffer [100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.0, 0.5% Nonidet P-40, 1 mM DTT, 100 U/ml RNasin RNase inhibitor (cat. no. N2511; Promega), 2 mM vanadyl ribonucleoside complexes solution (cat. no. 94742; Fluka BioChemica), 25 µl/ml protease inhibitor cocktail for mammalian tissues (cat. no. P8340; Sigma–Aldrich)]. After centrifuging, the cell lysate at 16 000 g for 15 min at 4°C, the supernatant was divided in three aliquots and pre-cleared twice by adding 50 µl of equilibrated protein A-agarose beads to each aliquot (50% agarose slurry in polysome lysis buffer) and rotating at 4°C for 1 h. After a brief centrifugation to remove beads, the supernatant from each Petri dish was divided in two aliquots and incubated with anti-ß-catenin antibody (2 µl/g) or aspecific rabbit IgG (2 µl/g) as a negative control. After an overnight incubation at 4°C, 50 µl of protein A-agarose beads were added and the samples were incubated for additional 4 h. Samples were briefly centrifuged to collect beads, which were washed four times with polysome lysis buffer. At this step, two beads aliquots were processed in order to perform a western blot for the presence of ß-catenin and HuR and the other aliquots were resuspended in polysome lysis buffer containing 0.1% sodium dodecyl sulfate and 30 µg proteinase K. Samples were incubated in a heating block at 50°C for 30 min. Extraction and analysis of COX-2 mRNA was performed by RT–PCR as described above.

In situ L-DOPA staining
L-DOPA (L-3,4-dihydroxyphenylalanine) has been shown to be a key substrate of tyrosinase (38). Cells were stained in situ using the L-DOPA reaction for tyrosinase activity. Briefly, cells were rinsed twice in 0.1 M sodium phosphate buffer pH 6.8, fixed for 20 min in 4% paraformaldehyde, washed three times with phosphate buffer and incubated at 37°C for up to 18 h in the dark with 5 mM t-DOPA (Sigma) in 0.1 M sodium phosphate buffer, pH 6.8. After incubation, the cells were rinsed with distilled water, fixed for 20 min in 4% paraformaldehyde and photographed by light microscopy.

Tyrosinase assay
After treatment, cells were washed twice with ice-cold PBS and resuspended in lysis buffer (10 mM Tris–HCl, pH 7.4, 120 mM NaCl, 25 mM KCl, 2.0 mM EGTA, 1.0 mM EDTA, 0.5% Triton X-100 and protease inhibitor cocktail) in a precooled tube. Following 45 min of incubation on ice, the cell lysates were centrifuged at 13 000g for 15 min at 4°C to obtain the supernatants. The tyrosinase activity in the cell lysates was determined by measuring the oxidation of DOPA to DOPA chrome (38). Briefly, the reaction mixture (200 µl) containing cell lysates (20 µg protein) and 1.0 mM t-DOPA in 0.1 M sodium phosphate buffer (pH 6.8) was placed on a 96-well microplate and incubated at 37°C for 60 min. The DOPAchrome formation was spectrophotometrically determined at 490 nm.

Statistical analysis
The results were expressed as the means ± standard error of the means. Two-tailed unpaired t-test was adopted to determine significant differences between groups in Supplementary Figure S1 and S3 (available at Carcinogenesis Online) and Figure 1E and F. One-way analysis of variance was used to determine significant differences among groups in Figures 1(A–D), 2, 3, 4, and 6.

Fig. 1. Effect of DHA on the expression of COX-2 protein and mRNA and on COX-2 mRNA stability in WM115 and WM266-4 melanoma cells. (A and B) Expression of COX-2 protein in WM115 and WM266-4 melanoma cells treated for 24 h with increasing DHA concentrations. Below: one representative of three similar western blot analyses is shown. Above: data are the means ± SEMs of three different experiments. Values not sharing the same superscript are significantly different (P < 0.05, one-way analysis of variance, followed by Tukey’s test). (C and D) COX-2-actin mRNA ratio in melanoma cells treated with 30 µM DHA for increasing times. Values are expressed as means ± SEMs of triplicate assays. Values not sharing the same superscript are significantly different (P < 0.05, one-way analysis of variance, followed by Tukey’s test). (E and F) COX-2 mRNA stability in WM115 and WM266-4 cells treated with 30 µM DHA for 60 min after ActD addition and COX-2 mRNA was analyzed by RT–PCR (see Materials and Methods). Data are the means ± SEMs of three different experiments. *Significantly different from control at the same time point (E: P < 0.05, F: P < 0.04, two-tailed unpaired t-test).
When significant values were found ($P < 0.05$), post hoc comparisons of means were made using Tukey’s honestly significant differences test. All the statistical analysis was performed using GraphPad InStat 5 Software (GraphPad Software, San Diego, CA).

**Results**

The basal expression or activity of proteins related to melonocytic differentiation or the acquisition of malignancy were evaluated in both the primary and metastatic melanoma cell lines WM115 and WM266-4 (Supplementary Figure S1 is available at Carcinogenesis Online). We evaluated the expression of COX-2, which has been associated to apoptosis resistance in melanoma (8), and the activity of tyrosinase, the key enzyme in melanin synthesis and a marker of melanocytic differentiation (38). The metastatic cell line showed significantly higher levels of COX-2 and lower tyrosinase activity, indicative of a more malignant phenotype. In agreement, they showed also a lower Bax/Bcl-2 protein ratio, suggestive of their decreased basal susceptibility to undergo apoptosis.

Figure 1 shows that the constitutive expression of COX-2 protein (Figure 1A and B) was inhibited in a dose-dependent manner by 10–30 μM DHA in both the cell lines treated for 24 h, even though the effects were more marked in the primary WM115 cells (with 30 μM DHA: 92% inhibition in WM115 cells versus 69% in WM244-6 cells, $P < 0.05$). DHA also inhibited COX-2 mRNA expression (Figure 1C and D) in both the cells, again more in WM115 cells (by 69%) than in WM244-6 cells (by 50%; $P < 0.05$). Moreover, the degradation of COX-2 mRNA was accelerated by the presence of 30 μM DHA in cells whose mRNA transcription was inhibited by 10 μg/ml actinomycin D. One hour after the addition of actinomycin D, the cellular level of COX-2 mRNA decreased by 27 and 37% in the DHA-treated WM115 and WM266-4 cells, respectively ($P < 0.05$), indicating that the DHA treatment was able to reduce the stability of COX-2 mRNA (Figure 1E and F).

DHA also inhibited the growth (Supplementary Figure S2 is available at Carcinogenesis Online) of both the cell lines. The effect was concentration dependent, observed earlier (also at 24 h, data not shown) and more conspicuous in the WM115 cell line than in the WM266-4 cells (IC$_{50}$ for DHA at 48 h: 38.3% higher in WM266-4 cells than in WM115 cells). Figure 2A shows that the treatment with DHA also caused the induction of apoptosis in both the melanoma cell lines in a concentration-dependent manner, even though the WM115 cells appeared to be more responsive than the WM266-4 cells. We observed that the DHA-induced apoptosis was caspase-3 dependent (Figure 2B) and was associated with an increase in Bax and a decrease of Bcl-2 protein expression that made the Bax/Bcl-2 ratio increase in both the cell lines (Figure 2C and D) in a DHA concentration-dependent manner. Moreover, we examined whether the pro-apoptotic effect was specific for DHA or also other fatty acids [EPA (20:5 $n$-3), linoleic acid (18:2 $n$-6) and oleic acid (18:1 $n$-9)] could induce apoptosis (Figure 2E). Besides DHA, we observed that only the other n-3 PUFAs, EPA (30 μM) was able to slightly, but significantly, induce apoptosis in both the cells, thus suggesting that the pro-apoptotic effect was specific for n-3 PUFAs.

Next, we investigated (Figure 3) the expression of HuR, a well-known stabilizer of COX-2 mRNA (29). Preliminary experiments had
shown that a 4 h treatment with 30 μM DHA reduced the total cellular expression of HuR in WM115 cells (∼53.5% reduction). We, however, investigated in detail and in both the cell lines the change of HuR expression in the cytosol (Figure 3A and B) since it is in this location that this protein exerts its mRNA-stabilizing effect. We observed that DHA reduced the expression of cytosolic HuR in a concentration- and time-dependent manner (maximal effect at 4 h, data not shown) in both the melanoma cells, even though the effect was prominent in WM115 cells (at 4 h with 30 μM DHA: 85 and 53% reduction in WM115 and WM266-4 cells, respectively). The DHA-induced decrease of cytosolic HuR was evident as early as 2 h after DHA addition (data not shown) but became significant only after 4 h. In concomitance, the expression of HuR in the nuclear fraction increased in both the cells (Figure 3C), and the effect was more evident in WM115 cells (ratio between the nuclear expression of HuR and that of the nuclear marker protein SRNP70 increased by 105.1% in WM115 cells and by 99.5% in WM266-4 cells). The immunocytochemical analysis of HuR (Figure 3D) in WM266-4 cells confirmed that DHA (30 μM) induced HuR to move from the cytosolic location (the preferential location in basal conditions) to a prevailing nuclear position, where its mRNA-stabilizing role cannot be exerted. To support the involvement of HuR in the regulation of COX-2 expression in melanoma cells and in their susceptibility to undergo apoptosis, we decreased HuR expression using another strategy. We silenced HuR expression by transfecting WM266-4 cells with the specific siRNA (Figure 4), obtaining the maximal inhibitory effect on HuR expression after 72 h (81% inhibition). We observed that the inhibition of COX-2 expression obtained with the HuR silencing at 72 h did not differ (P > 0.05) from that obtained in control cells treated during the last 24 h with 30 μM DHA (for a total incubation of 72 h). In these conditions, we found that both HuR siRNA transfection and DHA treatment induced apoptosis (126 and +115% apoptosis increase in HuR siRNA and DHA-treated cells, respectively).

Recently, it was found that β-catenin could act in cooperation with cytosolic HuR to carry out COX-2 mRNA stabilization in colon cancer cells (35). Thus, we investigated whether β-catenin could be related to the HuR-mediated stabilization of COX-2 mRNA also in melanoma cells. We found that β-catenin co-immunoprecipitates with HuR in WM266-4 cells (Figure 5A and B) and that the HuR/β-catenin complex also binds to COX-2 mRNA (Figure 5C and D), suggesting a role for β-catenin in the stabilization of COX-2 mRNA in melanoma cells. We also observed that the addition of 30 μM DHA reduced the expression of total (Figure 6A and B) β-catenin in both the cell lines studied. Moreover, we showed that in WM266-4 cells DHA specifically reduced the expression of cytosolic β-catenin (Figure 6C) and the level of the HuR/β-catenin complex (by 29%, data not shown). In concomitance, DHA enhanced the β-catenin expression in the nuclear fraction of WM266-4 cells (Supplementary Figure S3 is available at Carcinogenesis Online) as demonstrated by the conspicuous increase (by about six times) of the ratio between the expression of nuclear β-catenin and...
the nuclear protein marker SRNP70. The morphological analysis of β-catenin-immunostained WM266-4 cells revealed that DHA also enhanced β-catenin nuclear expression (Figure 6D), suggesting that DHA caused the translocation of most of the residual β-catenin to the nucleus. An increased number of granules (apparently melanosomes) was also evidenced in WM266-4 cytosol after DHA treatment. To identify the exact nature of these granules, we performed the L-DOPA assay by incubating WM266-4 cells with L-DOPA (1 mM), a substrate for tyrosinase in one of the key steps of the melanogenic pathway (38), and by analyzing the formation of DOPA chrome, an intermediate product in the melanogenic pathway (38) (Figure 6E). Before DHA treatment, the WM-266-4 cells did not show any DOPA chrome-stained granules (melanosomes) in their cytosol, confirming the scarce differentiation degree of this metastatic cell line. However, the treatment with DHA induced the formation of a remarkable number of melanosomes in the cytosol, suggesting that DHA induced melanogenesis. To support this hypothesis, we investigated the effect of DHA on the activity of tyrosinase, the key enzyme in the melanocytic pathway, and found that in both the cells DHA significantly increased the activity of the enzyme (Supplementary Figure S4 is available at Carcinogenesis Online).

Discussion

In this study, we have observed for the first time that the constitutive expression of COX-2 in melanoma cells may be related to the stabilization of its mRNA by the mRNA-binding protein HuR. We observed higher expression of COX-2 and cytosolic HuR in the metastatic WM266-4 melanoma cell line than in the primary WM115 cells and that expression was inversely related to the ability of the melanoma cells to undergo apoptosis. Moreover, we found that following treatment of the cells with the specific siRNAs for HuR, the levels of COX-2 decreased and apoptosis was induced, suggesting the involvement of HuR and COX-2 in the regulation of apoptosis in these cancer cells. DHA, one of the major n-3 PUFAs in fish oil, was able also to inhibit COX-2 expression and cytosolic localization and protein abundance of HuR in these cells as well as to decrease COX-2 mRNA stabilization and to induce apoptosis, indicating that the HuR-mediated stabilization of COX-2 may represent a target of DHA in melanoma cells. We also observed that β-catenin was physically associated with HuR and COX-2 mRNA in these cells. The treatment with DHA reduced β-catenin total and cytosolic expression and its binding to HuR and induced the translocation of the residual β-catenin to the nucleus. Moreover, we observed for the first time that DHA stimulated specific features of melanocytic differentiation in these cells and hypothesized that a relationship may exist between the prevalent nuclear localization of β-catenin induced by DHA in melanoma cells and the pro-differentiating effect of this fatty acid. COX-2 is an inducible enzyme involved in prostaglandin synthesis during inflammation (39). Its constitutive expression plays a functional role both in the development and progression of melanoma (7,9). We have observed herein that, in line with their metastatic origin, the WM266-4 cell line expresses higher levels of COX-2 than the WM115 primary cell line. Moreover, WM266-4 cells show a lower basal propensity to undergo apoptosis, as indicated by their relatively lower Bax/Bcl-2 protein ratio. Moreover, WM266-4 cells showed lower basal activity of tyrosinase, a key enzyme in the melanogenic pathway and a marker of melanocytic differentiation (38). All these findings concur to demonstrate the higher malignity of the metastatic WM266-4 cell line and are in agreement with previous findings.
showing their high expression of integrin subunit α-V, which is related to the invasive and metastatic potential (40), and relatively low levels of caveolin-1, a suppressor of melanoma metastasis (41).

The treatment of WM115 and WM266-4 cells with DHA decreased their protein and COX-2 mRNA expression, inhibited growth and induced apoptosis in both the cells, even though WM115 cells were always more sensitive to the action of DHA. Nevertheless, all the effects also occurred in the metastatic WM266-4 melanoma cell line at a relatively low (10–30 μM) fatty acid concentration (42), suggesting the high therapeutic potential of this fatty acid in advanced stage melanoma, where apoptosis resistance represents a primary cause of treatment failure and has been related to tumor progression (43). We observed that DHA induced a caspase-3-dependent apoptosis, which involved an increase in the Bax/Bcl-2 ratio. The pro-apoptotic effect was highly specific for DHA and not observed with the other fatty acids oleic acid or linoleic acid, whereas a slight effect was observed with EPA, suggesting that the pro-apoptotic effect was specific for n-3 PUFAs.

The constitutive expression of COX-2 has been related to the increased stabilization of COX-2 mRNA by the mRNA-binding protein HuR in a series of cancers (44–48), and the cytosolic expression of HuR itself has been associated with the high stages of tumors (45). Since, to the best of our knowledge, the cytosolic expression of HuR, and its COX-2 mRNA stabilizing function have never been investigated in melanoma cells, we decided to explore whether the mRNA stabilization of COX-2 mediated by HuR could be also involved in the regulation of COX-2 expression in these cells. The observation that WM266-4 cells express higher levels of COX-2 than the primary cells, and show a more prominent expression of cytosolic HuR protein, suggests that a strict relationship exists between the expression of these two proteins. This hypothesis is further supported by the observation that cytosolic HuR protein co-immunoprecipitates with COX-2 mRNA, thus suggesting that HuR may mediate the stabilization of COX-2 mRNA in these cells. As a matter of fact, when we down-regulated HuR in WM266-4 cells through the transfection of the specific siRNA, we observed that the expression of COX-2 protein was also markedly reduced. In concomitance, we observed an induction of apoptosis, thus further confirming the hypothesis that the induction of apoptosis in these cells is related to the post-transcriptional regulation of COX-2 expression.

We hypothesized that a modulation of COX-2 mRNA stability could also be involved in the inhibitory effect exerted by DHA on COX-2 expression in melanoma cells as well as in its pro-apoptotic effect. We demonstrated that the addition of DHA markedly reduced the stability of COX-2 mRNA in both the melanoma cell lines. Correspondingly, the cytosolic HuR expression was also reduced, whereas its nuclear expression was augmented, even though all the effect were less pronounced in WM266-4 cells than in WM115 cells. These results are in line with our recent finding obtained in immortalized human keratinocytes (28). In that case, we showed that the resistance to UV-induced apoptosis, considered a crucial step in skin cell carcinogenesis (49), could be overcome either by knocking-down HuR using its specific siRNA or by treating the cells with DHA. In both cases, the high levels of COX-2 expression were also reduced. Moreover, since both the total and cytosolic expression of HuR

Fig. 5. Endogenous HuR and β-catenin specifically interact and physically bind COX2 mRNA. (A and B) Co-immunoprecipitation of HuR with β-catenin. Total cell lysates (TCLs) from WM266-4 cells were immunoprecipitated (IP) with anti-β-catenin rabbit antibody or aspecific rabbit IgG as a control (Ctrl). Proteins were visualized by western blot (WB) with either anti-HuR (panel A) or anti-β-catenin antibodies (panel B). Nonspecific bands corresponding to immunoglobulin heavy and light chains are indicated with asterisks. (C) Fluorescence versus cycle number diagram of RT–PCR with primers for COX-2 mRNA. The graph plots the amplification patterns of positive control (Ctrl+: HaCaT cells) and WM266-4 cell sample (obtained from immunoprecipitation, see Materials and Methods). (D) Melting curve analysis of PCR products. Melting peaks show the same melting temperature (Tm).
decreased in melanoma cells following a treatment with DHA, whereas the expression of this protein in the nucleus increased, it can be suggested that DHA is able to induce both a degradation and a nuclear translocation of HuR.

It was recently observed that cytosolic β-catenin could cooperate with β-catenin to stabilize COX-2 mRNA in NIH3T3 and 293T cells and in colon cancer cells (35). This is a new role for β-catenin that so far has been generally considered either a component of the cadherin cell adhesion complex or a transcriptional co-activator in the canonical Wnt signaling inside the nuclei (50). We have now observed for the first time that β-catenin complexed with HuR and that the complex HuR/β-catenin is bound to COX-2 mRNA, suggesting a role for β-catenin as COX-2 mRNA stabilizer also in these cells. Recently, Bachmann et al. (51) related the lack of nuclear β-catenin expression observed in scarcely differentiated melanoma cells to increased thickness and malignity. In line with this finding, Chien et al. (52) also demonstrated that elevated levels of nuclear β-catenin in both primary melanoma and metastases correlate with a reduced proliferation index and improved patient survival. We have found that DHA is able to considerably increase the levels of β-catenin in the nuclei of the metastatic WM266-4 melanoma cells, which express low nuclear levels of this protein. We observed that, in concomitance, the total and cytoplasmic levels of β-catenin were reduced by DHA. Thus, DHA may exert several distinct effects on β-catenin in melanoma cells. First, it reduces β-catenin total expression, in agreement to what observed previously by us and others in different kinds of cancer cells (18,21). This reduction is accompanied by a dramatic decrease of β-catenin in the cytosol, thus making it less available for its binding to HuR and COX-2 mRNA. Finally, DHA induces the low levels of residual β-catenin to massively translocate into the nucleus, where, according to Chien et al. (52), could induce the transcription of genes specifically related to melanocyte lineage differentiation, thus favoring the reversion to a less malignant phenotype. As a matter of fact, we observed that melanoma cells acquired a more differentiated phenotype when treated with DHA, with in increased activity of tyrosinase (a marker of induced melanogenesis) and of melanosomes containing DOPA-chrome, an intermediate product of melanogenesis. This differentiating effect of DHA is in keeping with that observed in cancer cells of different origin treated with this fatty acid (53,54). Interestingly, the pro-differentiating effect of DHA was morphologically evident after only 6 h, whereas 24–48 h were needed to observe the morphological pattern of apoptosis. This observation suggests that, even though the expression and/or location of the molecular factors (HuR and β-catenin) underlying apoptosis and differentiation are modified by DHA precociously (after 3–6 h), different intervals of time are needed to complete these processes.

![Figure 6](image-url)

Fig. 6. Effect of DHA on the expression of β-catenin and melanogenesis in WM266-4 cells. (A) The expression of total β-catenin was measured in WM266-4 cells exposed to increasing concentration of DHA for 4 h. (B) The expression of total β-catenin was measured in WM266-4 cells exposed to 30 μM DHA for increasing times. (C) The expression of cytosolic β-catenin was measured in WM266-4 cells exposed to increasing concentrations of DHA for 4 h. Above in (A–C), data are the means ± SEMs of three different experiments. Values not sharing the same superscript are significantly different (P < 0.05, one-way analysis of variance, followed by Tukey’s test). Below in (A–C) one representative of three similar western blot analyses is shown in each panel. (D) Intracellular localization of β-catenin protein in WM266-4 melanoma cells in the presence and in the absence of 30 μM DHA, evaluated by immunocytochemistry. (E) Melanogenesis was evaluated by incubating WM266-4 cells pretreated or not for 6 h with 30 μM DHA in the presence of 5 mM l-DOPA for additional 18 h and analyzing them under light microscopy (see under Materials and Methods for details).
Overall, these findings allowed us to relate for the first time the constitutive expression of COX-2 in melanoma cells to the stabilization of its mRNA mediated by HuR and suggest that also β-catenin may play a role in HuR-mediated COX-2 stabilization in these cells. The data demonstrate also that the HuR-mediated stabilization of COX-2 may represent a target of DHA action in melanoma cells, together with β-catenin, that, following DHA stimulation, decreases its cytosolic level, reduces its binding to cytosolic HuR and moves massively into the nucleus. Moreover, the results suggest that a relationship may exist between the increased nuclear location of β-catenin induced by DHA and the differentiating (melanogenetic) effect of this fatty acid in melanoma cells. On the whole, the findings add further support to the application of DHA in the prevention and therapy of melanoma.

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Supplementary material
Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/.

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33. Serini, S. et al. (2009) Cyclooxygenase-2 may represent a target of DHA action in melanoma cells, together with beta-catenin, that, following DHA stimulation, decreases its cytosolic level, reduces its binding to cytosolic HuR and moves massively into the nucleus. Moreover, the results suggest that a relationship may exist between the increased nuclear location of beta-catenin induced by DHA and the differentiating (melanogenetic) effect of this fatty acid in melanoma cells. On the whole, the findings add further support to the application of DHA in the prevention and therapy of melanoma.


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