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Apoptosis Induced by Oxaliplatin in Human Colon Cancer HCT15 Cell Line

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Abstract. *Background: Oxaliplatin (L-OHP), active in a wide range of human and animal tumours, also CDDP-resistant, possesses unique molecular characteristics of action. However, the mechanisms by which the damage induced by L-OHP triggers a death signal are not yet fully defined. Materials and Methods: After L-OHP treatment of the HCT15 human colon cancer cell line, apoptosis was evaluated by DNA laddering detection and by flow cytometry; the effect on specific caspase-3, -8 and -9 inhibitors, mitochondrial membrane permeability transition, cytochrome C release and expression of CD95 and CD95L were also assessed. Results: HCT15 cells underwent apoptosis when treated with all used drug concentrations (7-25 μM). Treatment of cells with L-OHP resulted in the activation of caspase-8, -9 and -3, in a mitochondrial membrane depolarisation, and in an increase of CD95 receptor and CD95 ligand levels. Conclusion: The results correlated well with the ability of L-OHP to induce apoptosis and give further insights into the mechanisms underlying the L-OHP-induced apoptosis of tumor cells.*

The DNA-damaging platinum compounds are among the most frequently used drugs in the treatment of a variety of human malignancies. Oxaliplatin (trans-/-diaminocyclohexane oxalatoplatinum; L-OHP) was the first clinically available diaminocyclohexane platinum coordination complex and represents one of the few active drugs against human colorectal cancer (1-5). It has been shown to form DNA adducts differing from those determined by cisplatin (CDDP); indeed, its 1,2-diaminocyclohexane (DACH) carrier ligand is able to modify the N-Pt-N bond angle, resulting in bulky platinum-DNA adducts (3, 6). Preclinical data have shown that oxaliplatin is active in a wide range of human and murine tumour cell lines, and has been found to be non-cross-resistant with cisplatin in various cisplatin-resistant cell lines and tumours (3, 6, 7). However, the mechanisms by which the damage induced by this agent triggers a death signal are not yet fully defined.

In a recent study of our group, the apoptotic effect of L-OHP was compared with that of CDDP in two different human colon cancer cell lines, HCT116 and HCT15 (8). Both these cells are characterized by defects in the DNA mismatch repair system, being HCT116 hMLH1 mutant and HCT15 hMSH6 mutant (9); furthermore, while HCT116 express a wild-type p53 (wtp53), HCT15 cells contain a heterozygous p53 status (wt/mutant) (9). The susceptibility of these cells to undergo apoptosis after drug treatment appeared to be quite different. HCT116 cells, which express a wild-type p53 gene, were sensitive to both drugs, although the apoptotic effect of L-OHP appeared to be greater than CDDP. HCT15 cells, containing a mutant p53 sequence, underwent apoptosis when treated with L-OHP at all used concentrations (7-25 μM) in a dose-dependent manner, while they appeared to be insensitive to CDDP.

In the present work the role of caspase activity, mitochondrial membrane depolarisation and mitochondrial cytochrome C release in L-OHP-induced apoptosis of HCT15 cells was investigated. Secondly, the role of the CD95 system on drug-induced apoptosis as well as the ability of L-OHP to increase tumor cell susceptibility to undergo CD95-mediated apoptosis were explored.

Materials and Methods

Cell system and drug treatment. The human colorectal adenocarcinoma cell line HCT15 (ATCC CCL 225) was maintained in a 5% CO_2 atmosphere at 37˚C in RPMI 1640 medium, supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 μM glutamine and antibiotics. Cells were treated with L-OHP (SANOFI WINTHROP, Gentilly Cedex, France) at a concentration range of 7-25 μM for different times (2-24 h).

Apoptosis evaluation by DNA laddering detection. Cells ($1x10^6$), after the indicated treatments, were harvested, washed and incubated in 0.3 ml of 10 mM Tris-HCl pH 8.0 containing 25 mM EDTA, 100 mM NaCl, 0.5% SDS and 0.1 mg/ml proteinase K at 37˚C for 18 h.

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After phenol-chloroform extraction, the DNA was ethanol precipitated and resuspended in TE (10 mM Tris-HCl pH 8.0 and EDTA 1 mM), incubated for 1 h at 37˚C with 1 μg/ml RNAse, applied on a 1.8% agarose gel containing ethidium bromide (0.5 μg/ml), electrophoresed for 2 h at 100 V and photographed under ultraviolet illumination.

Apoptosis evaluation by propidium iodide solution. Apoptosis was measured by flow cytometry as described elsewhere (10). After culturing, the cells were centrifuged and the pellets were gently resuspended in 1.5 ml hypotonic propidium iodide solution (PI, 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma, St. Louis, MO, USA). The tubes were kept at 4˚C in the dark overnight. The PI-fluorescence of individual nuclei and the percentage of apoptotic cell nuclei (sub-diploid DNA peak in the DNA fluorescence histogram) were measured by flow cytometry with standard FACScan equipment (Becton Dickinson, San José, CA, USA). Where indicated, the effects of Z-DEVD-FMK (Calbiochem, Darmstadt, Germany), a caspase-3 inhibitor, Z-IETD-FMK (Calbiochem), a caspase-8 inhibitor and Z-LEHD-FMK (Calbiochem), a caspase-9 inhibitor, were investigated on L-OHP-induced apoptosis. In general, cells were incubated with the inhibitors for 30 min prior to the addition of L-OHP, at the concentrations indicated in the figure legends.

Caspase activities. Caspase activities were measured in cell lysates from not-treated and L-OHP-treated cells after a 6-h culture. The activity of caspase-9 was measured with a colorimetric assay kit (MHC-6/Caspase-9 Colorimetric Protease Assay Kit, Chemicon International, Inc. Temecula, CA, USA) according to the manufacturer's instructions. Absorbance measurements were made with a 96-well plate reader at 405 nm. The activities of caspase-8 and -3 were measured with colorimetric assay kits (ApoAlert Caspase-8 and ApoAlert Caspase-3 Assay Kit, Clontech Laboratories Inc. Palo Alto, CA, USA), according to the manufacturer's instructions. Absorbance measurements were made with a 96-well plate reader at 405 nm. Where indicated, the effect of Z-DEVD-FMK (Calbiochem), a caspase-3 inhibitor, Z-IETD-FMK (Calbiochem), a caspase-8 inhibitor and Z-LEHD-FMK (Calbiochem), a caspase-9 inhibitor, were investigated on L-OHP-induced caspase activity. In general, cells were incubated with inhibitors for 30 min prior to the addition of L-OHP, at the concentrations indicated in the figure legends.

Mitochondrial membrane permeability transition. Cells ($5x10^5$/ml), previously treated with L-OHP (25 μM) for 2 h, were incubated with 10 μg/ml JC-1 (Molecular Probes) for 15 min in the dark at room temperature, then washed twice with cold PBS and immediately analysed by flow cytometry. JC1 forms red fluorescent J-aggregates (590 nm) at higher $\Delta\Psi m$ and green fluorescent monomers (527 nm) at low-membrane potential. Changes in $\Delta\Psi m$ were, therefore, evaluated by the shift in fluorescence emission.

Cytochrome C release. Cells ($5x10^7$/ml), previously treated with L-OHP (25 μM) for 2 h, were harvested by centrifugation at 500xg for 5 min. Cytoplasmic extracts were prepared as previously described (11). The buffer included 20 mM HEPES (pH 7.5 with KOH), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 01 mM PMSF, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin and 25 μg/ml calpain inhibitor I. Protein concentrations varied from 2-5 μg/μl of extracts (Bio-Rad protein assay with BSA as the standard). Sample extracts (20 μg/ml) were loaded onto a 1.5% SDS-polyacrylamide gel and electrophoresed at 130 V for 2 h, then transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) at 100 V over a further 2 h. Membranes were blocked in 50 mM Tris (pH 7.5) with 500 mM NaCl, 1% BSA and 5% non-fat dried milk. The membranes were then probed with a purified mouse anti-cytochrome C monoclonal antibody (Pharmingen, San Diego, CA, USA; 1:500) in an identical solution, followed by peroxidase-labeled anti-mouse antibodies (1:10,000) and visualised by ECL (Amersham Biosciences, Buckinghamshire, UK).

Analysis of CD95 and CD95L expression. The expression of CD95 and CD95L protein was assessed by flow cytometry, using specific anti-human CD95 and anti-human CD95L mAbs. Following a 4-h drug treatment, HCT15 cells ($1x10^6$) were washed twice with PBS, fixed for 5 min at room temperature with paraformaldehyde (4% in PBS) and incubated at 4˚C for 60 min with 10 μg/ml anti-human CD95 mouse monoclonal IgM Ab (clone CH-11; Upstate Biotechnology, Inc., Lake Placid, NY, USA) or 10 μg/ml of irrelevant mouse IgM (Sigma) in PBS containing 1% fetal bovine serum and 0.5 μM EDTA. The specificity of the primary antibody was verified by Western immunoblotting and was determined to detect a Mr 45,000 CD95 molecule in whole-cell extracts of the colon tumor cell lines (not shown). After two washings with PBS, the cells were incubated for 30 min at 4˚C with constant rotation with 20 μg/ml of affinity-purified FITC-conjugated goat-antimouse IgG plus IgM (DAKO, Milan, Italy) in PBS containing 1% FBS and 0.5 μM EDTA. After two more washings, the cells were resuspended in PBS and analysed by flow cytometry with a FACscan cytofluorimeter (Becton Dickinson, San José, CA, USA). The fluorescence background was calculated in the absence of the primary Ab. The same procedure was applied for surface CD95L expression. Antihuman CD95L IgG_1 mouse monoclonal antibody (clone NOK-1, Pharmingen, San Diego, CA, USA) was used as primary antibody. Also for CD95L, the specificity of primary antibody was tested by Western immunoblotting and was determined to detect a Mr 40,000 CD95L molecule in whole-cell extracts of HCT15 cells (not shown). Where indicated, in order to analyse total CD95L levels, cells were previously permeabilized with 0.05 Nonidet P-40 in PBS before immunofluorescence.

Blockage of CD95 signaling with anti-CD95 neutralizing-antibody. Cells were treated for 24 h with L-OHP (25 μM) plus 0.5 μg/ml of an apoptosis-neutralizing monoclonal CD95 antibody (clone ZB4; Upstate Biotechnology, Inc., Lake Placid, NY, USA) or 0.5 μg/ml control antibody (mouse IgG, Sigma-Aldrich, Milano, Italy). Apoptotic cells were counted by flow cytometry after staining with PI as described above.

Drug-induced sensitivity to CD95-mediated apoptosis. For the CD95 sensitivity assay, cells were pretreated with L-OHP (25 μM) for 4 h, washed in fresh medium and treated with 0.5 μg/ml anti-human CD95 mouse monoclonal IgM Ab (CH-11, Upstate Biotechnology) or 0.5 μg/ml irrelevant mouse IgM antibody (Sigma) for another 18 h and then analysed for percentage of apoptotic cells by flow cytometry of PI staining, as described above.

Statistical analysis. The data were statistically analysed using a Student's *t*-test and significant differences between the means were calculated as *p* values, $p<0.05$ being considered statistically significant.

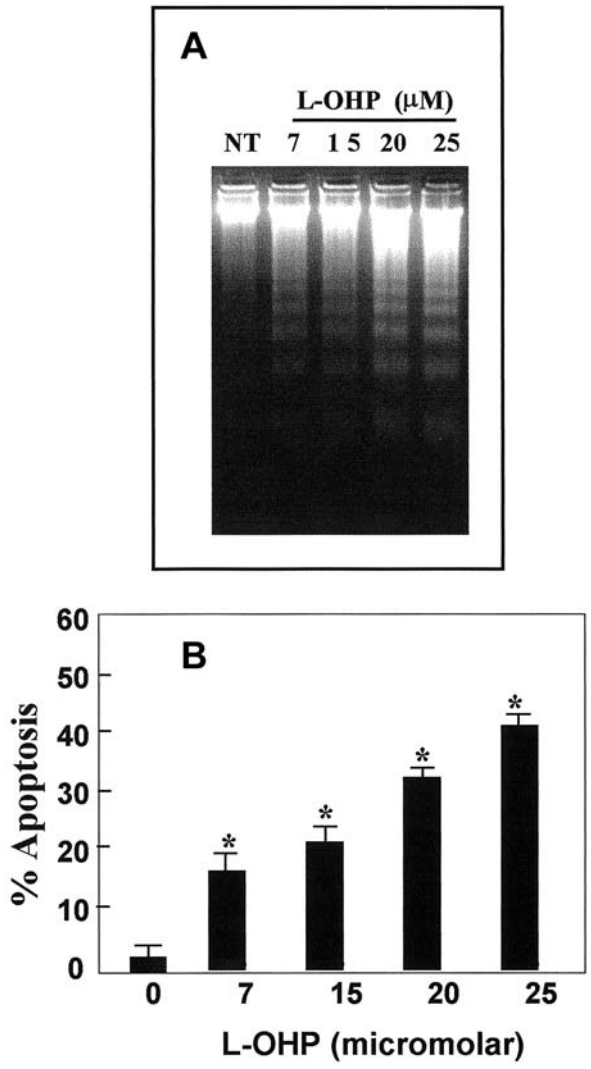

Figure 1. *(A) Internucleosomal DNA fragmentation in HCT15 cells after treatment with L-OHP. Cells were cultured in the presence or absence of drugs at indicated doses for 24 h. Ethidium bromide-stained agarose gels of electrophoresed DNA are shown. The results are representative of 3 independent experiments. (B) Apoptosis in HCT15 cells after treatment with L-OHP. Apoptosis was quantified by FACS analysis 24 h after the addition of drug at different doses (7-25 μM) by measuring the percentage of the cell population with subdiploid DNA content. Mean values±SD of percent subdiploid population obtained from 4 independent experiments are shown. All values marked with * were significantly different from control values (p<0.001).*

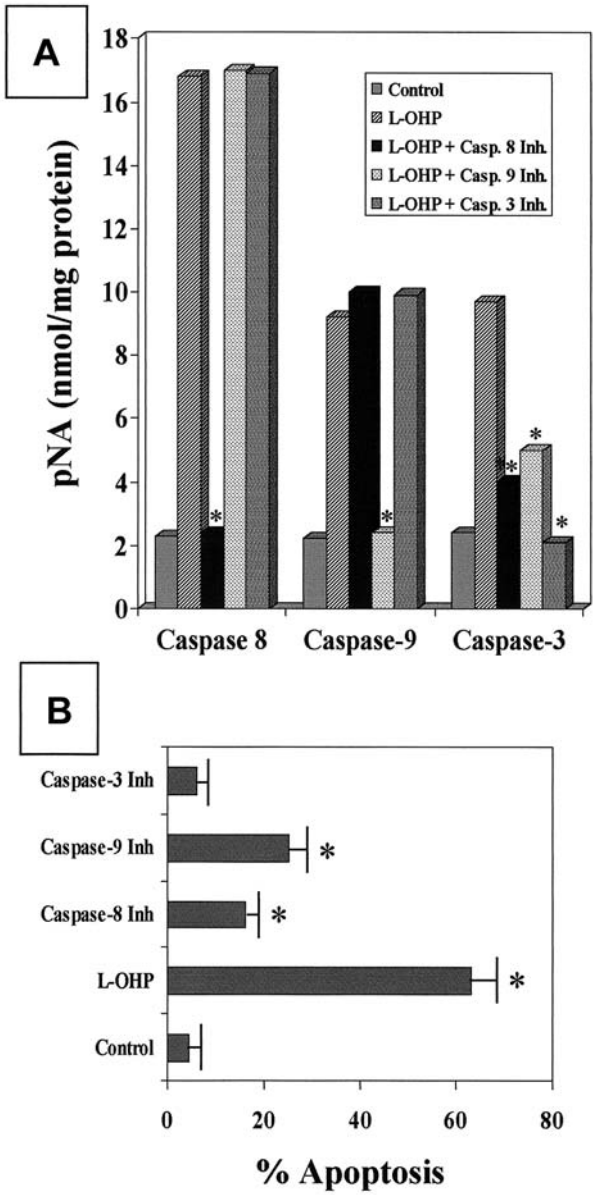

Figure 2. *Caspase activation by L-OHP treatment. (A) L-OHP-induced caspase activity: effect of inhibitors. HCT15 cells were treated for 8 h at 37°C with L-OHP (25 μM). Cell lysates were assayed for caspase-8, -9 and -3 by using an assay based on spectrophotometric detection of the chromophore pNA after cleavage from the specific-labeled substrates IETD-pNA, LEHD-pNA and DEVD-pNA, respectively. Caspase activity is expressed as nmol pNA/mg protein. Mean values of three different experiments in duplicate are reported (SE<5% of mean values; * = p<0.001 as compared to L-OHP-treatments). (B) Effect of caspase-8 inhibitor (Z-IED-FMK, 75 μM), caspase-9 inhibitor (Z-LEHD-FMK, 75 μM) and caspase-3 inhibitor (Z-DEVD-FMK, 100 μM) on L-OHP-induced apoptosis of HCT15 cells after 18 h culture. Mean values±SE of three different experiments in duplicate are reported (* = p<0.001 as compared to control values).*

Results

L-OHP-induced apoptosis. The ability of L-OHP (7-25 μM) to induce apoptosis in HCT15 cells was investigated first. The analysis of DNA fragmentation by DNA laddering analysis indicated that no detectable apoptosis was present in not treated cells (NT) and that the treatment for 24 h with L-OHP was able to induce apoptosis in HCT15 cells (Figure 1A). Drug-induced apoptosis was quantified by cytofluorimetric analysis of DNA content upon PI staining.

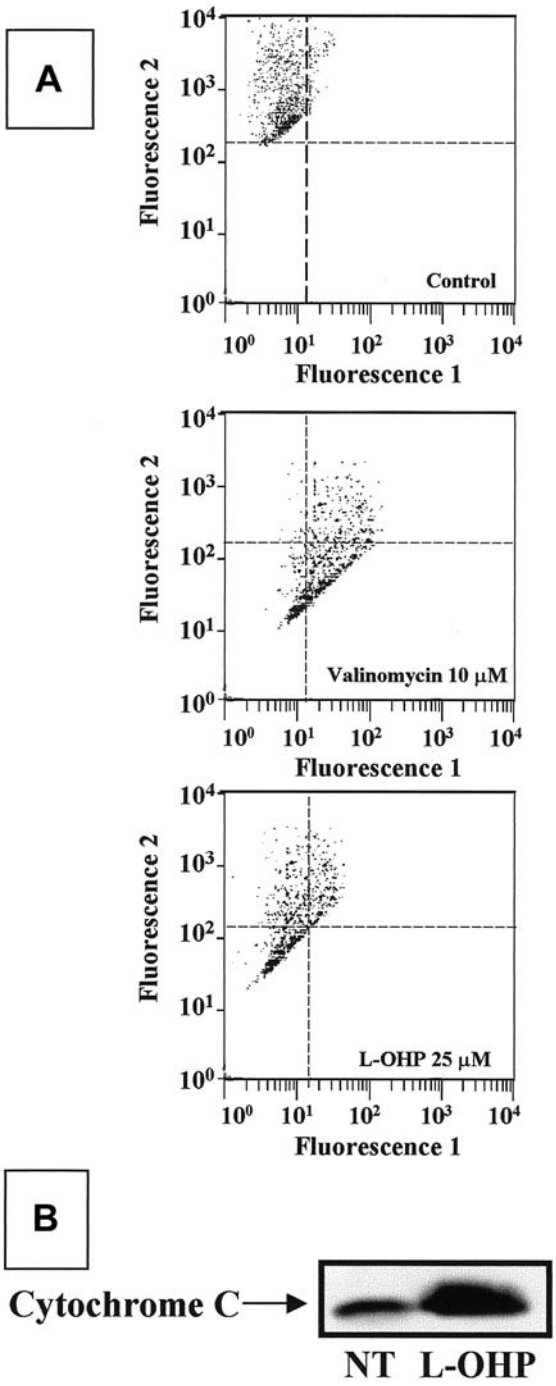

Figure 3. *L-OHP-induced cytochrome C release (A) and mitochondrial membrane potential loss (B).*

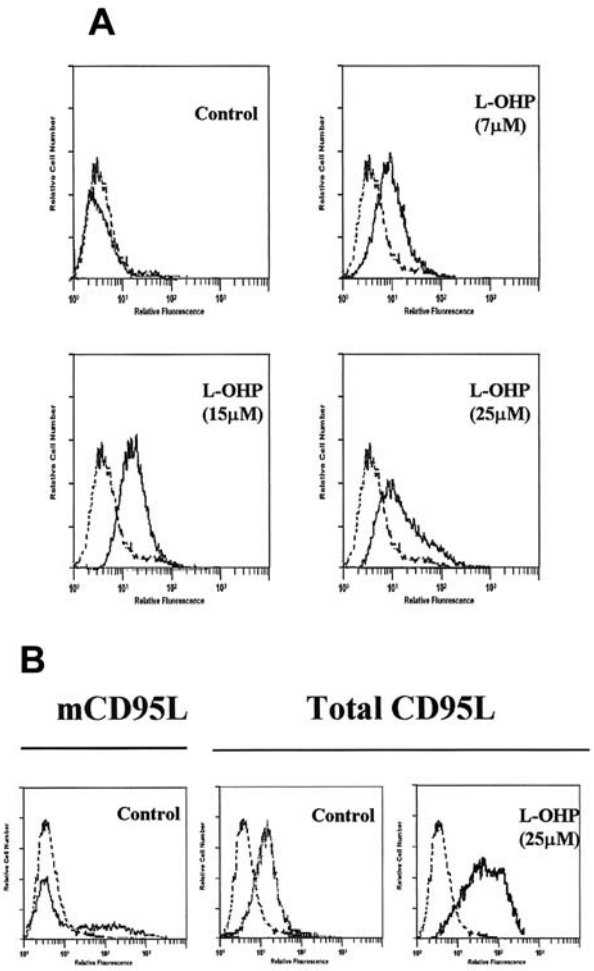

Figure 4. *(A) FACS analysis of CD95 receptor expression in HCT15 cells before (control) and after 4 h treatment with L-OHP at the indicated concentrations. The profiles are from one representative of 3 independent experiments. Dotted lines represent staining with secondary antibody alone. (B) FACS analysis of membrane-bound CD95L (mCD95L) or total (intracellular and membrane-bound) CD95L in unpermeabilized and permeabilized HCT15 cells, respectively. The effect of 4 h treatment with L-OHP (25 μM) on total CD95L is also shown. The profiles are representative from 3 independent experiments. Dotted lines represent staining with secondary antibody alone.*

L-OHP treatment was able to induce a dose-dependent apoptosis, which was also statistically significant at 7 μM ($p<0.001$ when compared to untreated cells) (Figure 1B).

L-OHP-induced caspase-8, -9 and -3 activation. To evaluate the caspase role in the L-OHP-induced HCT15 cell apoptosis, we tested the effect of L-OHP treatment on the activity of caspase-8, -9 and -3 using specific substrates. As a further control, we also used the specific inhibitor for each caspase tested. The results indicate that L-OHP activated caspase-8, -9 and -3 (Figure 2A). Experiments with caspase inhibitors suggested that L-OHP treatment induced two parallel caspase pathways: sequential activation of caspase-8 and -3 and sequential activation of caspase-9 and -3.

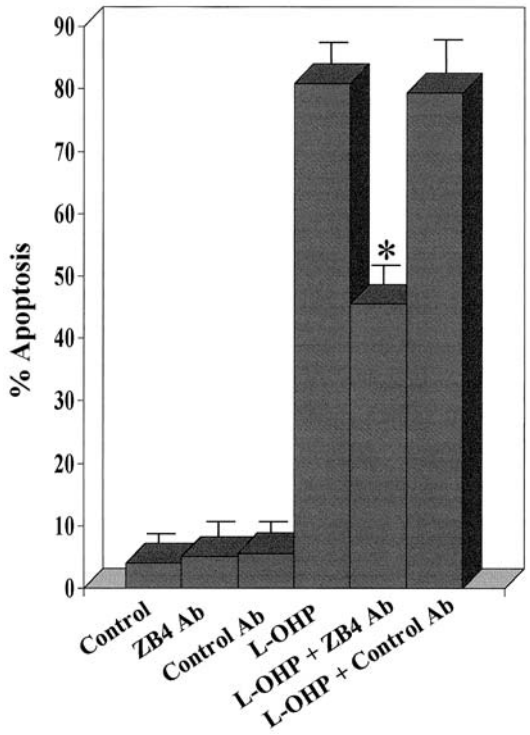

Figure 5. *Effect of antagonistic anti-CD95 antibody on L-OHP-induced apoptosis in HCT15 cells. Cells were pre-incubated or not for 1 h in the presence of 2 μg/ml ZB4 antagonistic antibody and then left untreated or treated with 25 μM CDDP or L-OHP for 24 h. The percentage of apoptotic cells was determined by PI staining and flow cytometry. Results are expressed as the means of 3 independent experiments±SD. The value marked with * was significantly different from that relative to L-OHP alone ($p<0.001$).*

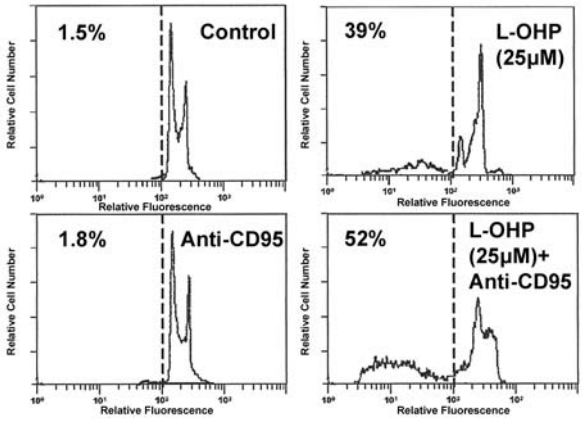

Figure 6. *Increased responsiveness towards induction of apoptosis by CD95 receptor stimulation after treatment with L-OHP. Cells were untreated or treated with drugs (25 μM) for 4 h, washed and incubated in fresh medium in the presence or absence of agonistic anti-CD95 Ab (10 μg/ml) for an additional 18 h. Flow cytometric profiles of DNA content upon PI staining from a representative experiment are shown. Two other independent experiments gave similar results.*

Indeed, while caspase-3 inhibitor was able to abrogate only caspase-3 activity, caspase-8 and -9 inhibitors, while they did not modify caspase-9 and caspase-8, respectively, partially but significantly reduced caspase-3, caspase-8 and caspase-9 activity. Moreover, specific caspase-8 and -9 inhibitors (respectively Z-IETD-FMK and Z-LEHD-FMK) were able to partially but significantly inhibit L-OHP-induced apoptosis ($p<0.001$ *vs.* L-OHP treatment) (Figure 2B). On the other hand, treatment with the caspase-3 inhibitor (Z-DEVD-FMK) totally abrogated L-OHP-induced apoptosis ($p<0.001$ *vs.* L-OHP treatment) and caused an apoptosis level not significantly different from control. These results indicate that both caspase-8 and -9 activation, through different pathways, led to the effector caspase-3 activity and may contribute to L-OHP-induced HCT15 cell death.

L-OHP induces mitochondria potential transition (ΔΨ) and cytochrome C release. It has been reported that mitochondria membrane potential transition (ΔΨ) and cytochrome C release are involved in apoptosis induction (12, 13). We performed experiments to evaluate the possible effect of L-OHP treatment on ΔΨ. For that purpose, HCT15 cells were treated with L-OHP and ΔΨ was evaluated at 2 h. As shown in the Figure 3A, L-OHP was able to induce mitochondrial potential transition comparable to that induced by valinomycin, used as control. This activity correlated well with the release of cytochrome C in the cytoplasm (Figure 3B). These results indicate that L-OHP-induced apoptosis may require the activation of a number of biochemical events including mitochondrial membrane potential transition and cytochrome C release.

Effects of L-OHP on CD95 receptor and CD95L expression in HCT15 cell lines. The role of the CD95 system in L-OHP-induced apoptosis in HCT15 cells was investigated. The possible induction or increased expression of CD95 and CD95L in HCT15 cells was evaluated by immunofluorescence and cytofluorimetry after a 1 to18-h incubation in the absence or presence of L-OHP at different doses (7, 15 and 25 μM). The results indicated that HCT15 cells showed no detectable presence of surface CD95 molecules. The treatment with L-OHP was able to significantly increase, in a dose-dependent manner, the CD95 expression in HCT15 cells. This effect was already evident at 1- h drug-treatment, reached a peak at 4-h and persisted at the same level up to the maximum treatment period (18 h). In Figure 4A are reported the results obtained at 4-h treatment from a representative experiment. Two other experiments gave similar results. In all experiments an

irrelevant IgM control Ab was employed as negative control and no positive signals were observed with it (not shown).

To analyse the membrane-bound CD95L (mCD95L) expression on HCT15 cells, phenotypic analysis of unpermeabilized cells by flow cytometry was performed using an anti- human CD95L mouse monoclonal Ab, as described in Materials and Methods. A significant percentage of HCT15 cells (30-40%) express high levels of CD95L on their surface. In Figure 4B are reported the results from a representative experiment. Two other experiments gave similar results. Drug treatment did not significantly influence the mCD95L expression at any employed dose (not shown). On the contrary, the total (intracellular plus membrane-bound) CD95L expression analysis, performed on permeabilized cells, showed that CD95L, constitutively present in HCT15 cells, appeared to be strongly up-modulated in a dose-dependent manner by L-OHP. One representative from three experiments showing the effect of the drug at 25 μM concentration is shown in Figure 4B. In all experiments an irrelevant IgG1 control Ab was employed as negative control and no positive signals were observed with it (not shown).

Effect of blocking antibody anti-CD95 on drug-induced apoptosis. To determine whether drug-induced tumor cell death and/or cell cycle arrest could involve interaction between CD95 receptor and CD95L, we used the antagonistic ZB4 anti-CD95 antibody. HCT15 cells were treated for 24 h with L-OHP at 25 μM in the absence or presence of 2 μg/ml ZB4 anti-CD95 blocking antibody, added to the culture medium 1 h before drug exposure. In these conditions, the ZB4 antibody was able to significantly inhibit apoptosis induced by L-OHP (38-42% apoptosis inhibition; $p<0.001$ *vs.* L-OHP-treated cells) (Figure 5).

Pretreatment of CD95-resistant HCT15 cells with L-OHP renders them CD95-sensitive. The basic susceptibility of HCT15 cells to undergo apoptosis after crosslinking of CD95 receptor through an agonistic monoclonal antibody anti-CD95 was analysed. HCT15, not showing a detectable CD95 receptor expression, appeared to be resistant to apoptosis in the presence of anti-CD95 Ab, as expected. In Figure 6 are reported the cytofluorimetric profiles relative to a representative experiment. Of note, up-regulation of CD95 receptor expression in HCT15 cells after a 4-h L-OHP pre-treatment correlated well with a significant increase of responsiveness towards CD95-mediated apoptosis.

Discussion

In the present work, we analysed the apoptotic effects of L-OHP on the human colon cancer HCT15 cell line. Our results indicate that L-OHP was able to induce, in a dose-dependent manner, apoptotic cell death in these cells, as determined by DNA laddering and cytofluorimetric analysis of hypodiploid nuclei. L-OHP-induced apoptosis appeared to also be statistically significant at the lower concentration used (7 μM). The mechanisms underlying the L-OHP-induced apoptosis of HCT15 cells were also investigated. In particular, the critical elements of drug-induced apoptosis, including activation of activator and effector caspases and mitochondrial function with concomitant release of apoptogenic factors (*i.e.* cytochrome C) were analysed. A wide number of signals and stimuli have been described to participate in the induction and inhibition of apoptosis so that the survival of a specific cell is under the control of a wide complex of signals. As an example, between the enzymatic pathways, caspase activities have been shown to be important regulators of programmed cell death (14-21). The possible role of caspases, including the co-ordinated activation of caspase-8 and -3 or -9 and -3, has been described in a variety of experimental systems (13, 19-21). Molecules, including those of the bcl-2 family, are important regulators of programmed cell death and it has been shown that their activity is, in part, involved in the regulation of mitochondrial functions including cytochrome C release (12, 22). Caspase activation has been shown in most apoptosis models and two main pathways: caspase-8 and -3 or caspase-9 and -3 activation have been proposed (23). In particular, L-OHP-induced apoptosis, here described, correlates with activation of both these pathways. The sequential activation of caspase-9 and –3 has been mainly associated with the release of cytochrome C, the latter being able to stimulate the binding between apoptotic protease activating factor-1 (Apaf-1) and dATP, thus inducing the formation of a multimeric Apaf-1, cytochrome C complex, apoptosome, able to activate pro-caspase-9 (24). Our results indicate that L-OHP was able to induce mitochondrial membrane potential loss and cytochrome C release in cytosolic fraction of HCT15 cells, which correlated well with caspase-9 and -3 activation. On the other hand, the sequential activation of caspase-8 and -3 has been mainly associated with the triggering of death receptor family members, which include CD95 receptor. The CD95 receptor/CD95 ligand (CD95/CD95L) system is known as one of the key regulators of apoptosis (23). Engagement of CD95 by agonistic anti-CD95 antibodies triggers programmed cell death in a variety of cell types (25-28). The induction of CD95 receptor expression in cancer cells exposed to chemotherapeutic drugs, including cisplatin, has been reported to correlate with wt p53 status by some authors (29) and to be p53-independent by others (30). On the other hand the anticancer drug-induced CD95L up-regulation has been shown to be independent of the tumor cell p53 status (29, 30). In the attempt to analyze the role of the CD95 system in L-OHP-induced apoptosis in our cell system, the expression of both CD95 receptor and ligand were analysed before and after drug treatment. L-OHP was able to induce, in a dose-

dependent manner, the CD95 receptor expression in HCT15 cells. With regards to the cellular distribution of CD95L, as reported for other cell types (31, 32), we observed in HCT15 cells a major intracellular localization of CD95L molecules. Even though the physiological meaning of this cellular distribution is still unclear, it is worth noting that an uncharacterized metalloproteinase cleaves the 40-kD membrane-bound CD95L to generate the 26-29 kD-soluble fragment (33-35). The real contribution of soluble CD95L in the apoptosis of CD95-positive cells still remains to be established. Indeed, various data would suggest that soluble CD95L has, *per se*, a very weak apoptotic activity and is able to antagonize the apoptosis of membrane-bound CD95L, which has been indicated as the functional form of CD95L (35-38). To this end, of particular interest are the observations of Martinez-Lorenzo *et al.* showing the ability of activated human T cells to release bioactive CD95L and APO2 ligand in microvesicles as intact nonproteolyzed molecules (39). In our hands, drug treatment did not significantly influence the membrane-bound CD95L expression at any employed dose. On the contrary, the intracellular CD95L expression analysis showed that CD95L, constitutively present in HCT15 cells, appeared to be strongly up-modulated in a dose-dependent manner by L-OHP. Experiments with the antagonistic ZB4 anti-CD95 antibody confirm that the L-OHP-induced apoptosis in HCT15 cells partially involved the CD95 system. Moreover, our results indicate that L-OHP-induced apoptosis of HCT15 could be associated, at least partially, with its ability to up-regulate the CD95 system in these cells. Finally, L-OHP was able to sensitize HCT15 to CD95-mediated cell death and this effect was associated with the induction of membrane CD95 expression. These results suggest that chemotherapy with L-OHP in patients affected by colon cancer with MMR deficiency and p53 inactivation could also lead to a increased sensitivity to CD95-mediated apoptosis by natural killer cells or tumor-specific T cytotoxic cells. This could support a role for this drug in the adjuvant setting and/or in association with immunomodulator therapies.

Our results provide new insights into the molecular determinants of chemosensitivity and apoptosis of tumor cancer cells to L-OHP. In particular, the apoptotic pathways include the loss of mitochondrial membrane potential and the cytochrome C release to cytosol, with the consequent activation of caspase-9 and -3. On the other hand, L-OHP treatment led to the up-regulation and activation of the CD95 system, probably with a consequent caspase-8 and -3 activation. Finally, the L-OHP-induced sensitization to CD95-mediated apoptosis could be a new application of immune therapy. The chemical structure of the L-OHP molecule could contribute to explaining the high sensitivity of MMR-deficient cell lines to this drug as well as the involvement of the CD95 system in L-OHP-induced apoptosis, independently of the genetic inactivation underlying the MMR deficiency or the p53 status.

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