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Effects of extra virgin olive oil phenols on HL60 cell lines sensitive and resistant to anthracyclines

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Abstract

The aim of our study was to evaluate the capability of a crude extract of phenols from extra virgin olive oil of Moraiolo cultivar to induce apoptosis and/or differentiation in sensitive and resistant HL60 cell lines to anticancer drugs (Typical Multidrug Resistance). Our data highlight that the crude extract is able to induce apoptosis on both sensitive and resistant cells, whereas the exposure to a number of anticancer drugs does not induce apoptosis in resistant cells. In differentiation experiments we investigated the capability of crude extract of phenols to induce the expression of CD11 granulocytic or CD14 monocytic cell surface antigen in sensitive and resistant HL60 cell lines. At IC50 dose level (17 μg/ml and 32 μg/ml respectively for sensitive and resistant cell lines), the crude extract induced differentiation associated with the expression of CD14 monocytic cell surface antigen either in sensitive or resistant cell lines but not that of CD11 granulocityc cell surface antigen. Further investigations are in progress to better clarify the mechanism by which olive oil phenols induce differentiation differentiation on this cell line.

Introduction

A number of *in vitro* studies highlight that the polyphenolic fraction of extravirgin olive oil contains several molecules which may induce direct cytotoxic effects on human tumour lines. Although the mechanisms by which these molecules may affect tumor cell growth are not fully understood, a number of experimental evidence suggests that these substance may exerts their antiproliferative effects by activating specific apoptosis pathways with a mechanisms which resemble those of antitumor agents (Della Ragione *et al.*, 2000; Fabiani *et al.*, 2006; Fabiani *et al.*, 2002.). Interestingly, the apoptotic pathways activated by molecules present in the polyphenolic fraction of extravirgin olive oil are those which result to be disregulated in cancer cells resistant to antineoplastic agents (Longley and Johnston, 2005). Several experimental indicated that caspases, enzymes belonging to the cystenil-proteinase family, play an important role in apoptosis activation (Oliver and Vallette, 2005). The intracellular activity of these proteases is regulated by IAPs proteins (apoptosis inhibiting proteins) that play a central role in the regulation of caspase-dependent apoptosis and in cell differentiation (Oliver and Vallette, 2005; Vucic and Fairbrother, 2007; Tamm *et al.*, 2004). The pharmacological modulation of the expression of apoptosis regulating proteins may be of clinical relevance in order to find out new and more effective and selective therapeutic strategies. On the basis of these considerations we have undertaken some investigations to evaluate the ability of a crude extract of phenols (CE) from extra virgin olive oil of Moraiolo Cultivar to induce apoptosis and/or differentiation in sensitive and resistant HL60 cell lines.

Materials and Methods

Cell culture and chemicals: Mouse anti-human CD14 and mouse anti-human CD11c were purchased from Becton Dickinson, Mountain View C.A. Human promyelocitic leukemia HL60 cells as parental (HL60/S) or resistant (HL60/R) variants were grown in RPMI and 10% FBS (GIBCO, BRL Gran Island, New York, U.S.A.) with 2mM glutamine (GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin).
The phenols crude extract was obtained from extra virgin olive oil of Moraiolo *Cultivar* as previously described (Gill *at*

al., 2005). The crude extract was dissolved in dimethyl sulphoxide. The content of phenolic fractions is reported in table 1.

Cells were plated at a density of $2x10^5$ cells/ml in 25 cm^2 flask and grown at 37° C in a humidified 5% CO2 atmosphere. The effects of, crude extract or Doxorubicin (DXR) on cell growth were assessed by seeding at a density of $2x10^5$ cells/ml in the presence of various concentrations of the drugs. After 48 hours, cells were counted by trypan blue exclusion.

Morphological evaluation of apoptosis. $2x10^5$ cells/ml were exposed to various concentrations of crude extract or to various drugs. After 24 hours or 48 hours cells were examined for induction of apoptosis by morphological evaluation as previously described (Simoni *et al.*, 1999).

Evaluation of expression of caspase 3

$2x10^5$ cells/ml were exposed to the IC_{50} of crude extract or to various drugs. After 24 hours cells were examined for expression of caspase 3 by flow cytometry (FACScan Becton Dickinson)

Analysis of cellular differentiation. Cells $2x10^5$ were exposed to the IC_{50} of crude extract and after 4 days were examined for induction of differentiation by morphology and flow cytometry as previously described (Simoni *et al.*, 1999).

Phenolic Compounds	mg/100mg Crude Extract
3,4-Dihydroxyphenyl Ethanol (3,4 DHPEA)	0.67
p-Hydroxyphenyl Ethanol (p-HPEA)	6.79
Dialdehydic form of elenolic ac. linked to 3,4 DHPEA	19.12
Dialdehydic form of elenolic ac. Linked to p-HPEA	22.21
(+)-1-acetoxipinoresinol and (+)-pinoresinol	3.32
Oleuropein aglycon (3,4-HPEA-EA)	15.76

Table 1 - *Content of Phenolic Compounds in crude extract of Moraiolo extra-virgin olive oil*

Results

Antiproliferative effects of Crude Extract.

The IC_{50} value of CE and DXR in the HL60 cell lines sensitive and resistant is reported in table 2.

DRUG	IC50° HL60S	IC50° HL60R	RI*
DXR	0.022 µM	0.60 µM	27
CE	17 µg/ml	32 µg/ml	1.8

Table 2 - *Drug sensitivity profiles of HL60/S and HL60/R Human leukaemia cell lines*

Data are the mean of at least four separate experiments. The standard deviation are less than 10%.

°IC50 is the dose inhibiting the cell growth by 50%

*RI= dose inhibiting the cell resistant growth by 50% / dose inhibiting the cell sensitive growth by 50%

Morphological evaluation of apoptosis

The effects of crude extract on the induction of apoptosis is reported in table 3. The concentration level able to induce 50% apoptosis in cells (AC50) was 20 µg/ml for sensitive cells and 45 µg/ml for resistant cells. The CE was able to induce apoptosis on both sensitive and resistant cells, whereas the exposure to a number of anticancer drugs did not induce apoptosis at all in resistant cells lines (fig. 1).

CE	% Apoptotic HL60/S cells	% Apoptotic HL60/R cells
10 µg/ml	5	6
17 µg/ml	36	6
20 µg/ml	50	6
25 µg/ml	96	6
32 µg/ml	-	20
45 µg/ml	-	50
50 µg/ml	-	66

Table 3 - *Effect of EG on induction of apoptosis in HL60/S and HL60/R Human leukaemia cell lines*

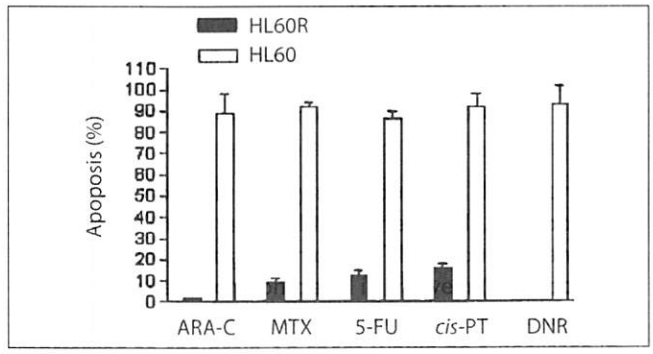


Fig. 1 - *Proapoptotic effect of cytosine arabinoside (Ara-C), metotrexate (MTX), 5-fluoruracil, (5FU), cis-platin (cis-Pt) and daunorubicin (DNR) on HL60/S and HL60/R*

Data are the mean of at least four separate experiments. The standard deviation are less than 10%.

Evaluation of expression of Caspase 3

The results reported in Fig. 2 showed that CE was able to induce activation and increased expression of caspase 3 in sensitive HL60 variant. No activation was observed in resistant cell line.

Induction of differentiation.

To evaluate the capability of CE to induce differentiation as shown by morphological examination (Fig. 1) and by the increase of expression CD14 antigen (Fig. 2), HL60/S and HL/60R were exposed to the IC_{50} concentrations of CE, namely, 17 µg/ml and 32 µg/ml respectively up to four days.

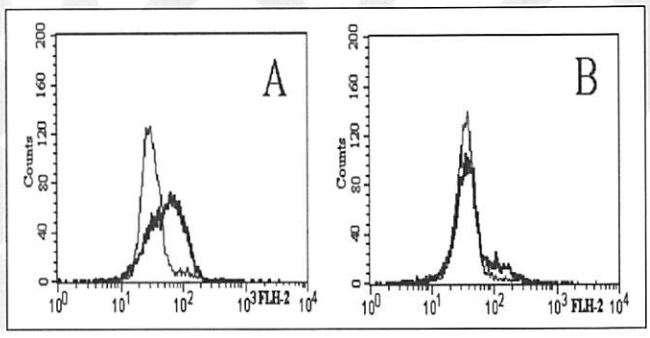
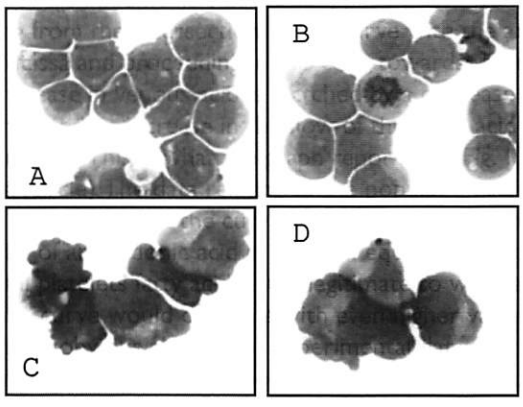


Fig. 2 - *Expression of caspase 3 in HL60/S (A) and HL60/R (B) after 24 hours of treatemant with the IC50 of crude extract of Moraiolo. Fine line control cells; Fit line treated cells.*

Fig. 3 - *Differentiating effects of Crude Extract (CE).*



A:HL60/S untreated cells; B:HL60/R untreated cells C: HL60/S cells exposed to CE for four days; D: HL60/R exposed to CE for four days.

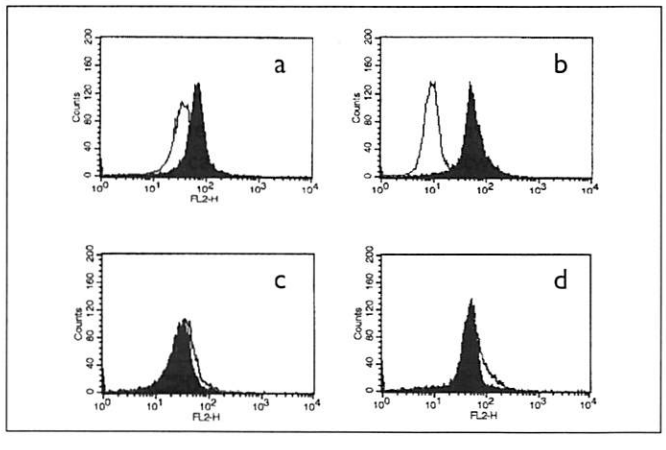


Fig. 4 - *Cytofluorimetric assay of the expression of differentiating cell surface antigen CD14 and CD11c on HL60/S and HL60/R cells after four days exposure to CE used at IC50 dose level. This figure is representative of at least three independent experiments. a) white histogram: CD 14 expression untreated HL60/S cells, black histogram CD 14 expression treated cells; b) white histogram: CD 14 expression untreated HL60/R cells, black histogram CD14 expression treated cells; c) white histogram: CD 11 expression untreated HL60/S cells, black histogram CD 11 expression treated cells; d) white histogram : CD 11 expression untreated HL60/R cells, black histogram CD11 expression treated cells.*

No expression of CD11c granulocytic cell surface antigen was observed in sensitive and resistant cell lines.

Discussion

The good sensibility to the cytotoxic effect of CE observed in HL60/S and HL60/R led us to investigate the mechanisms involved in the growth inhibitory effects and to assess whether the lower sensibility of resistant variant may be due to an escape from apoptosis. In line with the data reported by other studies (Fabiani *et al.*, 2008), our results suggest that CE is able to induce apoptosis on both sensitive and resistant cell line and that caspase 3 seems to be involved at least in the induction of apoptosis only in HL60/S but not in HL60/R. In the last cell lines CE could activate other caspase indepentent apoptotic pathways (Della Ragione *et al.*, 2000). In order to study the possible relationship between apoptosis and cell differentiation we assessed the capability of CE to induce differentiation in both cell lines. Our results showed that CE was able to induce differentiation as assessed by the expression of monocytic (CD14) cell surface antigen either in sensitive or resistant cell lines. As it has been reported that XIAP proteins are involved in the expression of monocytic CD14 cell antigen surface (Tamm *et al.*, 2004), further studies are in progress to assess the role of XIAP proteins in the modulation of CD14 surface antigen expression during the monocytic differentiation. In conclusion the good activity of CE on induction of apoptosis and differentiation of resistant variant could bring out new strategy in the treatment of resistant tumors. Futher studies in aimed at better assessing the therapeutic possibilities of CE against sensitive and resistant tumors are warranted by these observations .

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