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Levamisole induced apoptosis in cultured vascular endothelial cells

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1 To better understand the anticancer activity of Levamisole (LMS), which serves as an adjuvant in colon cancer therapy in combination with 5-Fluorouracil, this study analyses LMS' ability to induce apoptosis and growth arrest in cultured human micro- and macrovascular endothelial cells (ECs) and fibroblasts.

2 Cells exposed (24 h) to Levamisole (range: 0.5–2 mmol l^{-1}) alone or in combination with antioxidants (10 mmol l^{-1} glutathione or 5 mmol l^{-1} N-Acetylcysteine or 0.1 mmol l^{-1} Tocopherol) were evaluated for apoptosis (^{3}H-thymidine assays, *in situ* staining), mRNA/protein expression (Northern/Western blot), and proliferation (^{3}H-thymidine incorporation).

3 Levamisole dose-dependently increased apoptosis in ECs to 230% (HUVECs-human umbilical vein ECs), 525% (adult human venous ECs) and 600% (human uterine microvascular ECs) but not in fibroblasts compared to control cells (set as 100%).

4 Levamisole increased in ECs integrin-dependent matrix adhesion, inhibited proliferation (−70%), reduced expression of survival factors such as clusterin (−30%), endothelin-1 (−43%), bcl-2 (−34%), endothelial NO-synthase (−32%) and pRb (Retinoblastoma protein: −89%), and increased that of growth arrest/death signals such as p21 (+73%) and bak (+50%).

5 LMS (2 mmol l^{-1})-induced apoptosis was inhibited by glutathione (−50%) and N-Acetylcysteine (−36%), which also counteracted reduction by Levamisole of pRb expression, suggesting reactive oxygen species and pRb play a role in these processes.

6 The ability of LMS to selectively induce apoptosis and growth arrest in endothelial cells potentially hints at vascular targeting to contribute to Levamisole's anticancer activity.
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Keywords: Levamisole; endothelial cells; apoptosis; oxidative stress; tumor angiogenesis

Abbreviations: ECs, endothelial cells; ET-1, Endothelin-1; 5-FU, 5-Fluorouracil; GSH, Glutathione; HUMECs, human uterus microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; HVECs, adult human venous endothelial cells; ICAM-1, Intercellular adhesion molecule 1; LFA-1, Leukocyte function antigen-1; LMS, Levamisole; MHC, major histocompatibility complex; NAc, N-Acetylcysteine; ppET-1, preproET-1; pRb, Retinoblastoma protein; TGF-β, transforming growth factor β; Toc, Tocopherol

Introduction

Levamisole (LMS), an antihelmintic drug, is used in conjunction with 5-Fluorouracil (5-FU) as an adjuvant to avoid colon cancer relapse following its surgical resection (Moertel *et al*., 1995). Levamisole is thought to act mainly as an immunomodulator (Ferrini *et al*., 1994) and to cell type dependently (colorectal- or breast carcinoma, melanoma, leukemic cell lines and HUVECs) affect the expression of adhesion molecules (Kimball & Fischer, 1996) as ICAM-1 or LFA-1 as well as that of MHC Class I molecules. The exact mechanism of Levamisole's anticancer effect, however, is still unknown.

In vitro Levamisole seems to potentiate 5-FU's antiproliferative effect in different tumor cell lines (Kovach *et al*., 1992), possibly by modulation of phosphorylation processes relevant for both cell cycle progression and apoptosis. This applies also to endothelial cells (Yang *et al*., 1996), which have gained added actuality by the therapeutic potential of antiangiogenic action of angiostatin and endostatin (O'Reilly *et al*., 1994; 1997) and their ability to thereby impair tumor nutrition and growth. In that context it is of note that tumor growth depends in part on the balance between endothelial proliferation and apoptosis within the afferent tumor vasculature (Holmgren *et al*., 1995).

To better understand the impact of Levamisole on endothelial cells, apoptosis and associated processes were studied in detail in human umbilical vein, adult venous and microvascular endothelial cells and associated human fibroblasts. Apoptosis (Kerr *et al*., 1972; Wyllie, 1980; Baumgartner-Parzer, 1996) was evaluated by ^{3}H-thymidine assays and *in situ* staining as well as to associated gene/protein expression of Clusterin, ET-1, p53, p21, bcl-2, bak, bcl-xs, eNOS and pRb (Buttyan *et al*., 1989; Shichiri *et al*., 1997; Kastan *et al*., 1991; el-Deiry *et al*., 1994; Hockenbery *et al*., 1993; Nunez and Clarke, 1994; Berry *et al*., 1996). As oxidative stress is assigned an important role both as to apoptosis and endothelial dysfunction (Slater *et al*., 1996; Briehl & Baker, 1996; Baynes *et al*., 1991), the antioxidants Glutathione, N-Acetylcysteine and Tocopherol were analysed as to their ability to modulate levamisole mediated effects in vascular endothelial cells.

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Methods

Cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase treatment of umbilical veins (Jaffe *et al*., 1973) and cultured on gelatin-coated culture dishes (Baumgartner-Parzer *et al*., 1994) in Medium 199 (Sigma, St. Louis, U.S.A.) containing 20% foetal calf serum (FCS, Gibco, Grand Island, U.S.A.), 60 mg l^{-1} endothelial cell growth supplement (Technoclone, Vienna, Austria), penicillin-streptomycin (100 mg l^{-1}), fungizone (0.5 mg l^{-1}) and heparin (5×10^6 μ l^{-1}) as described previously (Baumgartner-Parzer *et al*., 1995a,b). The cultures were maintained at 37°C and 5% CO_2 and subcultures were performed using trypsin-EDTA. For all experiments endothelial cells were used as individual isolates in primary culture and first subculture. Endothelial cells were identified by typical phase contrast 'cobblestone' morphology and by immunofluorescence to von Willebrand factor antigen.

Human venous endothelial cells (HVECs) were isolated from excess superficial saphenous veins, harvested for bypass procedures. In brief, after incubation for 10 min of the inner surface with collagenase (Sigma, St. Louis, U.S.A.), the inner layer of endothelial cells was scraped with a scalpel blade into 35 mm gelatin-coated tissue culture dishes containing prewarmed medium (37°C) and were cultured as described for HUVECs.

Human uterus microvascular endothelial cells (HUMECs: as pools of at least five different donors) were purchased from Technoclone (Vienna, Austria) and treated according to the distributor's instructions. Due to their limited availability only passages 3 and 4 were used for experiments.

Fibroblasts were isolated from veins (FV) and arteries (FA) of human umbilical cords. After harvesting of the venous endothelial lining vessel tissue was minced into a tissue culture plate, prevented from floating by cover glasses and incubated in DMEM (Gibco BRL, Grand Island, U.S.A.) containing 10% FCS (Gibco), Penicillin-Streptomycin and fungizone (24) at 37°C and 5% CO_2. Vessel tissue and cover glasses were removed after growth of fibroblasts from the explants (usually 3–5 days) could be observed.

Exposure of ECs to Levamisole and antioxidants

Proliferating as well as confluent EC cultures or fibroblasts were exposed for 24 h (if not indicated otherwise) to Levamisole (0.25–5 mmol l^{-1}) in the absence and presence of N-Acetylcysteine (5 mmol l^{-1}) or Glutathione (10 mmol l^{-1}) or Tocopherol (0.1 mmol l^{-1}), all purchased from Sigma (St. Louis, U.S.A.).

Adhesion assay Selective integrin-dependent matrix adhesion (sensitive to chelation of divalent cations) was evaluated by treatment of confluent ECs with 1 mmol l^{-1} EDTA in PBS during rotation on an orbital shaker at RT after 15 and 30 min, respectively (Roth *et al*., 1993). Detached cells were counted and results expressed as per cent of total cells obtained by trypsinization (set as 100%).

^{3}H-thymidine proliferation assays Confluent HUVEC primary cultures were replated in flat bottomed 96-well tissue culture plates (15,000 cells $well^{-1}$) and allowed to adhere for 6 h. Subsequently, cells were exposed to ^{3}H-thymidine (final concentration: 1 μCi ml^{-1}) and the respective test agents as indicated in the Figures for 36 h, followed by two washing steps with PBS. After trypsinization and a freeze thaw cycle DNA incorporated ^{3}H-thymidine was trapped onto a glass fibre filter using a Betaplate TM 96-well-cell harvester (Wallac, Turku Finland). The amounts of ^{3}H-thymidine incorporated in control cells (without addition of levamisole) were set as 100% and the results expressed accordingly. All experiments were performed in quadruplicates.

^{3}H-thymidine apoptosis assays (Baumgartner-Parzer et al*., 1995a; Martz, 1993)*

Endothelial cells Semiconfluent plates (60 mm) of HUVEC primary cultures (0.5×10^6 cells) were labelled with ^{3}H-thymidine (1 μCi ml^{-1}, 3 ml per plate) for 36 h. Then the cells were rinsed twice with PBS, trypsinized and seeded into 24-well culture plates (6×10^4 cells $well^{-1}$).

Fibroblasts Fibroblasts were labelled with 5 μCi ^{3}H-thymidine for 36 h and treated as described for endothelial cells. After 24 h exposure of proliferating cells to Levamisole, N-Acetylcysteine or glutathione DNA fragmentation was quantitated as follows: 1 ml lysis buffer (20 mmol l^{-1} Tris, 4 mmol l^{-1} EDTA, pH 7.4, 0.4% Triton X-100) was added to each culture well (containing 1 ml of medium) and mixed by pipetting. One ml of the suspension was transferred to an Eppendorf tube, kept on melting ice for 10 min, centrifuged at $8000 \times g$ for 5 min at 4°C. Subsequently, fragmented radiolabelled DNA was counted in 500 μl of the supernatant using a 1900 TR Liquid Scintillation Analyser (Canberra Packard Meriden, CT, U.S.A.). Radioactivity of cells, treated with lysis buffer and 180 μg ml^{-1} DNAse (Boehringer Mannheim, Germany), was used as total activity. Results were expressed as fragmented DNA in per cent of total DNA. All samples were tested in triplicates.

In situ *staining*

After exposure of confluent cultures to Levamisole accelerated cell death was measured using the FragEI and Apop Tag *in situ* apoptosis kit (Oncor, Gaithersburg, MD), according to the manufacturer's instructions. Briefly, terminal deoxynucleotidyl transferase catalytically adds residues of digoxigenin-nucleotides to the 3′ hydroxyl ends (highly concentrated in apoptotic bodies) of double- or single-stranded DNA. The incorporated nucleotides form a random heteropolymer of digoxigenin-11-dUTP and ATP in a ratio that has been optimized for antidigoxigenin antibody binding. The antidigoxigenin antibody fragment carries peroxidase to the reaction site. Samples were counted in a blinded fashion under the microscope.

Northern blot analysis

Total cellular RNA was extracted from confluent HUVECs (4×10^6 cells) – after their incubation with levamisole and/or antioxidants – according to the method of Chomczynski & Sacchi (1987) using RNAzol (Cinna Biotecx, Houston, U.S.A.). Total RNA (20 μg) was electrophoresed on 1% formaldehyde agarose gels, tranasferred onto Hybond N nylon membranes (Amersham International, U.K.) and UV treated at 254 nm (5 min).

The p21, ppET-1 (kind gifts of J. Smith, Houston, and L. Casey, Dallas, U.S.A.), p53 and clusterin cDNA probe (ATTC, Rockville, U.S.A.) were labelled with ^{32}P-dCTP employing the Megaprime DNA labeling system (Amersham) and the 28srRNA oligonucleotide probe (Clontech Labora-

tories, Palo Alto, CA, U.S.A.), used to correct for loading inequalities, was labelled by the kinase reaction. Prehybridization and hybridization were carried out as described previously (Baumgartner-Parzer *et al*., 1995a).

Western blot analysis

Cell cultures incubated with the agents described above as well as their respective control cultures were rinsed twice with cold PBS, scraped from the dishes with cold lysis buffer (50 mmol l^{-1} HEPES pH 7.0, 0.5% NP-40, 250 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} NaF; protease inhibitors were added immediately before use: 0.2 mmol l^{-1} Na_3VO_4, 50 mmol l^{-1} β-glycerophosphate, 1 mmol l^{-1} PMSF, 2 mmol l^{-1} benzamidine, 10 μg ml^{-1} aprotinine, 10 μg ml^{-1} Leupeptin) on wet ice and immediately frozen in liquid nitrogen. After two freezing, thawing cycles including a centrifugation step ($15{,}000 \times g$ for 10 min at 4°C) aliquots of the supernatant, containing 10 ng protein (measured by the BCA-protein assay, Pierce, Rockford, IL, U.S.A.) were subjected to polyacrylamide gel electrophoresis. After a blocking step and incubation with primary (MoAbs mouse anti human bcl-2 and bak; rabbit anti human bcl-xs from Calbiochem (La Jolla, CA, U.S.A.); MoAb mouse anti human p53 and pRb from Santa Cruz (CA, U.S.A.); MoAb mouse anti human p21 and eNOS from Transduction Laboratories (Lexington, KY, U.S.A.)) and respective second antibodies (horseradish peroxidase conjugated sheep anti mouse and anti rabbit Ig (Amersham, U.K.)), the bands were visualized using the ECL (Amersham, Amersham Place, U.K.) or Super signal system from Pierce (Rockford, Illinois, U.S.A.).

Quantification of autoradiographies

Membranes from Northern and Western blot analyses were exposed to Kodak XAR5-Omat films. Densitometry was performed with a Hirschmann Elscript 400 densitometer (Analysentechnik Hirschmann, Munich, Germany) over multiple exposures to verify that readings were in the linear range.

Statistics

Data are expressed as means $\pm$ s.e.mean unless indicated otherwise. Statistical analysis was performed using a Wilcoxon signed rank test for mRNA expression data and paired Student's *t*-test with Bonferroni correction for cell numbers, ^{3}H-thymidine assays, *in situ* staining and Western blot data.

Results

Cell numbers

Mean cell numbers determined in confluent first subcultures of individual HUVECs isolates ($n=9$) were $(1.34 \pm 0.1) \times 10^6$ for control cultures and $(1.29 \pm 0.11) \times 10^6$ ($P=ns$), $(1.20 \pm 0.09) \times 10^6$ ($P<0.02$) and $(1.11 \pm 0.09) \times 10^6$ ($P<0.02$) for cultures exposed to 0.5, 1.0 and 2.0 mmol l^{-1} Levamisole for 24 h, respectively.

Apoptosis following exposure to Levamisole and antioxidants

Levamisole (LMS) dose-dependently increased apoptosis in HUVECs to 94% (0.5 mmol l^{-1} LMS), 118% (1 mmol l^{-1} LMS), 220% (2 mmol l^{-1} LMS) and 630% (5 mmol l^{-1} LMS) versus controls (set as 100%) as measured by ^{3}H-thymidine apoptosis assays (Figure 1). No such effect was observed for arterial and venous fibroblasts from umbilical cords (data not shown). Basal as well as Levamisole induced apoptosis were unaffected by Tocopherol, but reduced by the antioxidants GSH and NAc (Figure 2) by 50% (GSH) and 36% (NAc) at 2 mmol l^{-1} Levamisole.

In situ *staining of apoptosis in HUVECs, HVECs and HUMECs*

Endothelial cells exposed to Levamisole exhibited considerable vacuolization and increased rates of apoptotic cell death (Table 1A) as apparent by condensation of chromatin and the presence of apoptotic vesicles when compared to their intraindividual control cells without added Levamisole (Figure 3). Rates of apoptosis measured by *in situ* staining (1 mmol l^{-1} LMS:155 $\pm$ 16%, 2 mmol l^{-1} LMS:227 $\pm$ 33%, $n=5$, $P<0.05$) were slightly higher than those determined by ^{3}H-thymidine assays (1 mmol l^{-1} LMS:118 $\pm$ 6%, 2 mmol l^{-1}

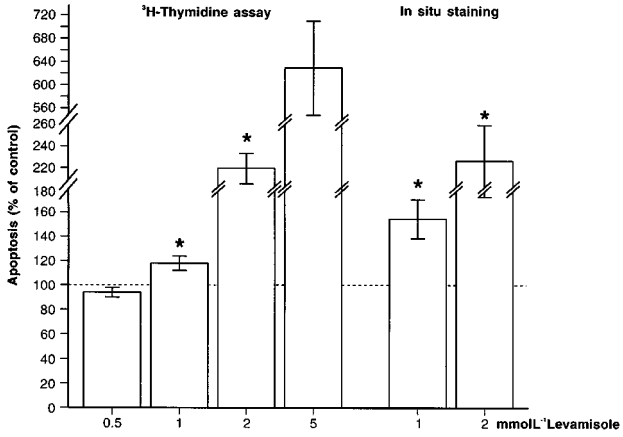

Figure 1 Effects of Levamisole on apoptosis in HUVECs as measured by ^{3}H-thymidine assays. HUVECs were exposed to 0.5, 1, 2 ($n=5$) and 5 mmol l^{-1} ($n=3$) Levamisole for 24 h. Effects of Levamisole were related to the respective intraindividual control cells (without addition of Levamisole) set as 100%. *$P<0.05$.

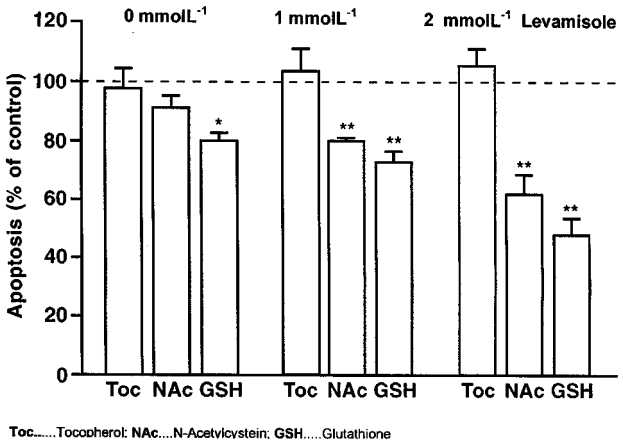

Figure 2 Modulation of basal and Levamisole-induced apoptosis in HUVECs ($n=5$) by the antioxidants Tocopherol (Toc), Glutathione (GSH) and N-Acetylcysteine (NAc), as measured by ^{3}H-thymidine assays. The effects of the antioxidants were related to their respective control cultures (basal and LMS-mediated apoptosis without addition of antioxidants) set as 100%. *$P<0.02$; **$P<0.005$.

Table 1 Effect of Levamisole on apoptosis and detachment in endothelial cells

A. Apoptosis[#]			*B. Detachment*[§]				
	Levamisole					*Levamisole*	
	1 mmol l^{-1}	*2 mmol l*$^{-1}$		*Detachment*†	*Control*	*1 mmol l*$^{-1}$	*2 mmol l*$^{-1}$
HUVECs ($n=5$)	155±16*	227±33*	HUVEC ($n=6$)	15 min	51±13	16±7*	4±0.7*
HVECs ($n=2$)	350±25	525±13		30 min	81±6	50±10*	22±11*
HUMECs (pool)		600					

[#]Percent of intraindividual control cells without added LMS, set as 100%, *$P<0.05$ versus control. [§]Detached cells in per cent of total trypsinized cells, set as 100%; control = intraindividual control cells without added LMS; †time of detachment; *$P<0.05$ versus control.

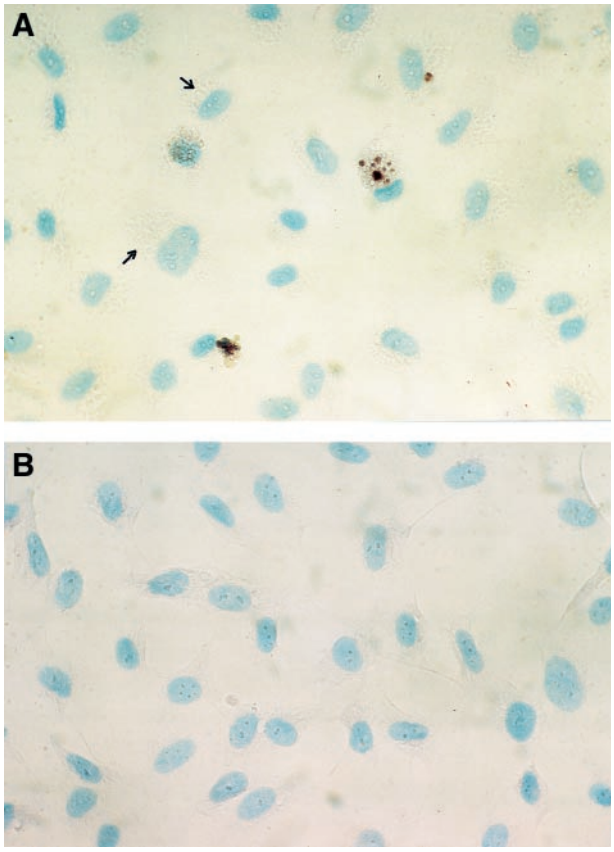

Figure 3 Effects of Levamisole on HUVECs' morphology (*in situ* staining). HUVECs exposed to (A) Levamisole (2 mmol l^{-1}) exhibited increased rates of apoptosis as apparent by condensation of chromatin and apoptotic vesicles (brown staining) as well as considerable vacuolization (arrowheads) compared to (B) control cells (without addition of Levamisole). Magnification ×400.

LMS:220±14%) for HUVECs. Peripheral (HVECs) and microvascular endothelial cells (HUMEC) exhibited higher rates of apoptosis (up to 600% of control) in response to LMS.

Adhesion assays (Table 1B)

Levamisole increased integrin-dependent matrix adhesion of endothelial cells and decreased their detachment from 51% (control) to 16% (1 mmol l^{-1} LMS) and 4% (2 mmol l^{-1} LMS) at 15 min incubation with EDTA. Similar results were obtained after 30 min agitation with EDTA.

Levamisole and antioxidants action on mRNA/protein expression in HUVECs (Figure 4)

Levamisole (2 mmol l^{-1}) reduced expression of mRNA for p53 (71%), clusterin (71%) and ET-1 (57%), but increased that of p21 (154%) compared to control cells (set as 100%). p53 and p21 mRNA expression was paralleled by that of the respective proteins (p53: 84%; p21: 173% of control). LMS differentially modulated protein expression of the members of the bcl-2 family in that bak was increased (150%), bcl-2 was decreased (66%), whereas bcl-xs (106%) was unaffected.

Maximum LMS (2 mmol l^{-1}) mediated reduction of hypophosphorylated pRb (65%) was restored by GSH but not N-Ac, whereas reduction of hyperphosphorylated (11%) pRb was counteracted by both antioxidants. Under basal conditions (without added Levamisole) both GSH and NAc exclusively increased the hyper-, but not the hypophosphorylated form of pRb (GSH: 212% NAc: 138% of control) (Figure 5).

In contrast, reduction by LMS (2 mmol l^{-1}) of eNOS expression (67.8±4.5% of control, $n=8$; $P<0.0005$) remained unaffected by these antioxidants (GSH: 72.3±7.9%; NAc: 66.0±6.9%).

^{3}H-thymidine proliferation assay

LMS dose-dependently reduced proliferation of HUVECs (1 mmol l^{-1} LMS: 71±6.8%; 2 mmol l^{-1} LMS: 29±4.8%; $n=4$; $P<0.05$) versus their intraindividual control cells (without added Levamisole), set as 100%.

Discussion

Levamisole selectively induces apoptosis in cultured vascular endothelial cells from different origins, but not in accompanying fibroblasts. This observation adds a new aspect to LMS's mode of action, which so far has been described as antiproliferative (Kovach *et al*., 1992; de Waard *et al*., 1998) and to affect both adhesion and MHC Class I molecule expression (Ferrini *et al*., 1994; Kimball & Fischer, 1996). The observed induction of endothelial apoptosis by Levamisole may in part explain its impact as an adjuvant in cancer treatment relating to vascular targeting (Denekamp *et al*., 1998; Schnitzer, 1998).

The observed growth arrest and apoptosis go along with a reduction by LMS of both the hyper- as well as the hypophosphorylated form of the cell cycle regulator pRb in endothelial cells. This observation suggests LMS to exert its proapoptotic action in endothelial cells *via* reduction in pRb as already described for other cell types (Berry *et al*., 1996). In that context it is of note, that sequestration of the transcription factor E2F by hypophosphorylated pRb represses – whereas E2Fs liberation by hyperphosphorylated pRb activates – transcription of genes required for DNA synthesis and cell cycle progression (Lopez-Marure *et al*., 1997; Spyridopoulos *et al*., 1998).

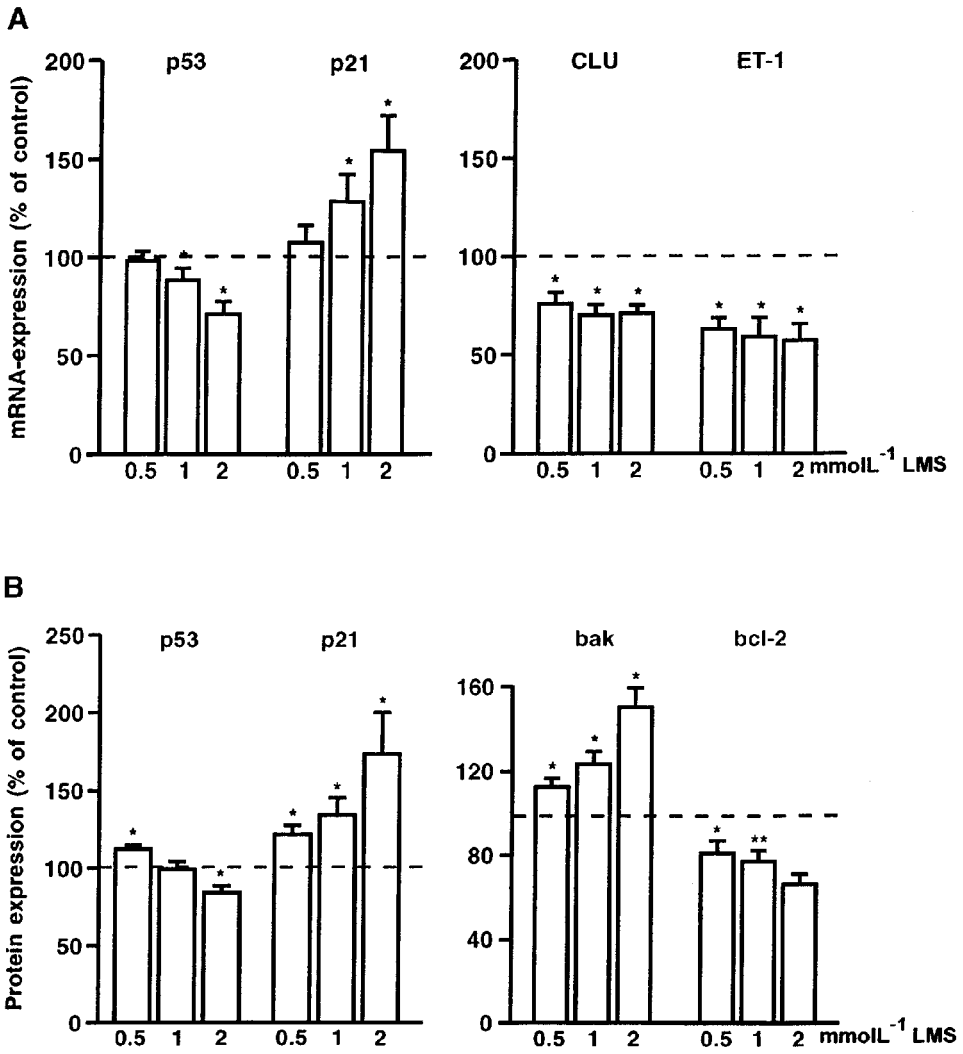

Figure 4 (A) mRNA expression of p53, p21, clusterin and ET-1 (Northern blot analysis) and (B) protein expression of p53, p21, bak and bcl-2 (Western blot analysis) in HUVECs ($n=7$) exposed to 0.5, 1 and 2 mmol l^{-1} LMS for 24 h compared to their respective intraindividual control cultures (without LMS) set as 100%. *$P<0.05$.

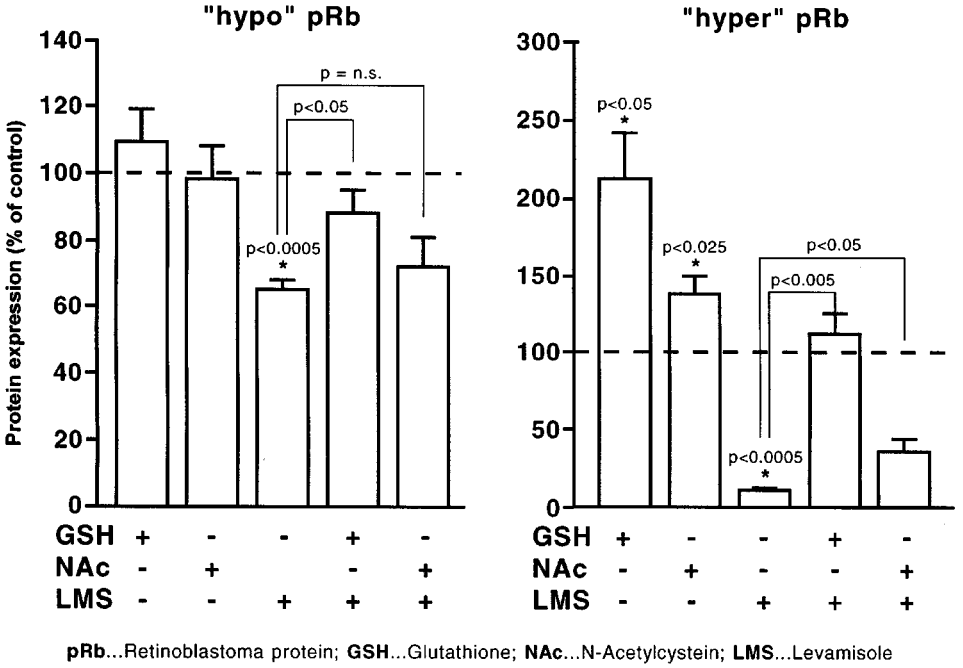

Figure 5 Changes in hypo- and hyperphosphorylated pRb (protein) in HUVECs ($n=6$) in response to Levamisole (2 mmol l^{-1}), GSH (10 mmol l^{-1}) and NAc (5 mmol l^{-1}) as measured by Western blot analysis. pRb expression in experimental cultures was related to that of their respective intraindividual control cultures (without addition of LMS) set as 100%.

As little is known about the molar ratios of pRb/E2F within a cell, one could only speculate that Levamisole-mediated loss of hyperphosphorylated pRb impairs release or availability of E2F (Spyridopoulos *et al*., 1998; Weinberg, 1996) and thus inhibits induction of genes necessary for cell cycle progression and/or cell survival.

Levamisole-mediated growth arrest and apoptosis could, however, also be attributed to increased protein expression of p21, an inhibitor of cyclin-dependent kinases (el-Deiry *et al*., 1994; Harper *et al*., 1993) and other potential death factors. Whereas p21 has been originally described to effectuate growth arrest following DNA damage after prior sensing by the tumour suppressor p53, LMS-induced endothelial apoptosis and associated p21 and bak upregulation apparently are p53-independent (Macleod *et al*., 1995). Taken together our data suggest a dominant role for pRb in the mediation of p53-independent LMS-induced apoptosis in endothelial cells similarly to that reported for other cell types (Nip *et al*., 1997).

In HUVECs Levamisole also increased bak, an apoptosis promotor, but decreased the apoptosis inhibitor bcl-2. Bcl-2 counteracts mitochondrial permeability transition (PT) – a central coordinating event of apoptosis and is closely related to cytoplasmatic vacuolization (Marchetti *et al*., 1996) as also induced by LMS.

As apoptosis in general and bcl-2 specifically have been linked to oxidative stress (Hockenberry *et al*., 1993; Kerr *et al*., 1972) it is of note that GSH and Nac, but not Tocopherol, inhibited Levamisole-induced apoptosis. The failure of tocopherol to counteract Levamisole action probably relates to its hydrophobicity and accumulation within the plasma membrane thereby forfeiting cytoplasmic antioxidative action. Since the intracellular GSH redox cycle is the major H_2O_2 detoxification system in endothelial cells, the observed inhibition by GSH of apoptosis suggests hydrogen peroxide plays a major role in LMS-induced endothelial growth arrest and apoptosis.

Levamisole reduced mRNA expression of clusterin, which only recently has been suggested to be a feature of surviving (French *et al*., 1994) not dying cells (Buttyran *et al*., 1989). Of note, clusterin (Apolipoprotein J) is closely associated with paraoxonase, believed to be responsible for the antioxidant activity of HDL (Mackness *et al*., 1997). Since increases of paraoxonase and clusterin are thought to represent a protective response to oxidative stress (41), reduction by Levamisole of clusterin expression in vascular endothelial cells may indicate reduced antioxidative capacity and increased vulnerability of these cells (Outinen *et al*., 1999) in response to other toxic agents.

In addition, decreased expression of ET-1 by LMS appears to support the notion of ET-1 being an endothelial survival factor (Shichiri *et al*., 1997).

As pRb was the molecule most markedly affected by LMS, interference by GSH and NAc was analysed, which both counteracted the reduction by Levamisole in the expression of hypo- and hyperphosphorylated pRb in HUVECs. From this it appears that endothelial apoptosis may also be affected *via* E2F, which is known to regulate genes important for cell death (Spyridopoulos *et al*., 1998). In contrast, LMS-dependent reduction in eNOS expression was not modulated by the antioxidants suggesting eNOS not to be involved in the regulation of LMS induced endothelial apoptosis and growth arrest. As eNOS, however, is a potent angiogenic factor (Murohara *et al*., 1998) its reduction may contribute to LMS' effects *in vivo*.

Besides promotion of apoptosis LMS's ability to increase expression or availability of endothelial adhesion molecules (integrins) – as shown in this study – may add to LMS' anticancer activity (Ferrini *et al*., 1994).

Since peak plasma concentrations after therapeutic Levamisole ingestion are only 10 μg ml^{-1} (0.08 mmol l^{-1}) and thus considerably lower than the concentrations (up to 2 mmol l^{-1}) used *in vitro* (Kovach *et al*., 1992; de Waard *et al*., 1998), it is of note that LMS is extensively metabolized *in vivo* and subject to a hepatic first pass effect (Kouassi *et al*., 1986). Thereby it may reach tissue concentrations considerably higher than those measured in plasma. In comparison with other *in vitro* models employing Levamisole concentrations up to 2 mmol l^{-1} (Kovach *et al*., 1992; de Waard *et al*., 1998) the rather short incubation period of 24 h in our study seems to primarily evaluate effects of the original substance, whereas incubation periods of 3 to 5 days (Kovach *et al*., 1992; de Waard *et al*., 1998) more readily cover effects of LMS metabolites.

In summary, this study demonstrates Levamisole-induced apoptosis and growth arrest to be associated with a loss of 'survival and antioxidative factors' (pRb, Clusterin, ET-1, bcl-2, eNOS) as well as with 'induction of growth arrest/death signals' (p21, bak). Such Levamisole-induced apoptosis seems to relate to oxidative stress which is counteracted by the antioxidants GSH and N-Ac and thus possibly depends on exhaustion of GSH and/or a concomitant increase of intracellular H_2O_2. Potentially, the observed effects of LMS on endothelial cell proliferation and apoptosis may hint at an angiostatic role of Levamisole supporting its anticancer activity.

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