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Mechanisms of resistance of normal cells to TRAIL induced apoptosis vary between different cell types

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Abstract Resistance of normal cells to tumour necrosis factor related apoptosis inducing ligand (TRAIL) induced apoptosis is believed to be mediated by expression of two decoy receptors. Here we show that the expression and localisation of TRAIL receptors (TRAIL-Rs) vary between different cells and that resistance to TRAIL is mediated by different mechanisms. The decoy receptor, TRAIL-R3, appeared important in protection of endothelial cells, whereas lack of surface death receptor expression and as yet unknown intracellular inhibitor(s) of apoptosis downstream of caspase-3 may play a major role in protection of melanocytes and fibroblasts from TRAIL induced apoptosis, respectively. Differential subcellular location of decoy receptors may be an important determinant of their effectiveness in different types of normal cells. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: TNF related apoptosis inducing ligand; TNF related apoptosis inducing ligand receptor; TNF related apoptosis inducing ligand induced apoptosis; Normal cell

1. Introduction

Tumour necrosis factor (TNF) related apoptosis inducing ligand (TRAIL) is a member of the TNF family which can induce apoptosis in a wide range of transformed cell lines but not normal cells [1,2]. TRAIL induces apoptosis by interaction with two death domain containing receptors referred to as TRAIL-R1/DR4 [3,4] and TRAIL-R2/DR5/TRICK 2 [5–7]. Both of these receptors are type I transmembrane proteins and their mRNAs were found to be widely expressed on normal tissues. The latter are believed to be protected from apoptosis by two additional receptors, TRAIL-R3/DcR1/TRID/LIT [8–11] and TRAIL-R4/DcR2/TRUNDD [12–14]. TRAIL-R3 is an extracellular glycosyl phosphatidylinositol (GPI) linked protein without an intracellular domain [8–11]. TRAIL-R4, like TRAIL-R1 and -R2, is a type I transmembrane protein but contains a truncated cytoplasmic death domain [12–14]. TRAIL-R3 and -R4 were thus considered to inhibit TRAIL induced apoptosis either by acting as decoy receptors or by providing inhibitory signals such as activation of the transcription factor NF-κB [14,15] which is known to regulate several inhibitors of apoptosis (c-IAP1, c-IAP2 and XIAP) [16,17]. In addition, the death receptors TRAIL-R1 and -R2 are also known to be able to activate NF-κB upon ligation [3–7]. A fifth TRAIL-R, osteoprotegerin, exists predominantly in a secreted form and appears to inhibit TRAIL induced apoptosis by competitive inhibition of TRAIL binding to the death receptors TRAIL-R1 and -R2 [18].

In studies on melanoma cell lines we have shown that TRAIL induced apoptosis is determined largely by the level of surface expression of the death receptors, particularly TRAIL-R2 [19]. Expression of the decoy receptors appeared to play very little role in determining their susceptibility to TRAIL [19]. We also found that intracellular pools of TRAIL receptors existed in most melanoma cell lines. The death receptors TRAIL-R1 and -R2 were primarily associated with the Golgi network, whereas the decoy receptors TRAIL-R3 and -R4 were localised predominantly in the nucleus [19,20]. Activation of NF-κB also appeared to have an important role in determining the susceptibility of certain melanoma to TRAIL induced apoptosis [21].

In the present studies we have attempted to examine more closely the basis of resistance to TRAIL induced apoptosis in normal cells. We show that the expression and location of TRAIL-Rs varies in different types of normal cells and that decoy receptor expression appears important in protection against TRAIL induced apoptosis in endothelial cells but not in melanocytes and fibroblasts.

2. Materials and methods

Human lung fibroblasts MRC-5 were obtained from Bio Whittaker Inc. (Walkersville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium containing 5% foetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Vic., Australia). Melanocytes were purchased from Clonetics (Walkersville, MD, USA) and cultured in medium supplied by Clonetics (Edward Kellar, Vic., Australia). Human umbilical vein endothelial cells (HUVECs) were kindly supplied by D. Clark (Transplantation Unit, John Hunter Hospital, Australia). HUVECs were isolated from umbilical veins of placenta by digestion in collagenase, as described elsewhere [22], and grown in M199 medium (Gibco, Australia) supplemented with 100 mg/l L-glutamine (Life Technologies), 20% FCS, 135 mg/l heparin (Sigma) and 16.7 μg/l endothelial cell growth supplement (Sigma). Over 95% of the

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Abbreviations: TNF, tumour necrosis factor; TRAIL, TNF related apoptosis inducing ligand; FasL, Fas ligand; TRAIL-Rs, TRAIL receptors; FLIP, FLICE inhibitory protein; IAP, inhibitor of apoptosis protein; HUVECs, human umbilical vein endothelial cells; GPI, glycosyl phosphatidylinositol; PI-PLC, phosphatidylinositol specific phospholipase C; ER, endoplasmic reticulum; WGA lectin, wheat germ agglutinin

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cells expressed CD31 and Von Willebrand factor tested by flow cytometry.

2.1. Monoclonal antibodies (mAbs) and recombinant proteins

Recombinant human TRAIL (lot 6321-19) prepared as described elsewhere [1] was supplied by Immunex (Seattle, WA, USA). Recombinant human Fas ligand (FasL) was kindly supplied as sterile supernatant by Immunex. The mAbs against TRAIL-R1 (IgG2a, M217), TRAIL-R2 (IgG1, M413), TRAIL-R3 (IgG1, M430) and TRAIL-R4 (IgG1, M444) were also supplied by Immunex [23]. Rabbit mAb against the active form of caspase-3 was purchased from Pharmingen (San Diego, CA, USA). Isotype control mAbs used were the ID4.5 (mouse IgG2a) mAb against *Salmonella typhi* supplied by Dr L. Ashman (IMVS, S.A., Australia), the 107.3 mouse IgG1 mAb purchased from Pharmingen (San Diego, CA, USA) and rabbit IgG from Sigma. The Golgi was identified with rhodamine labeled

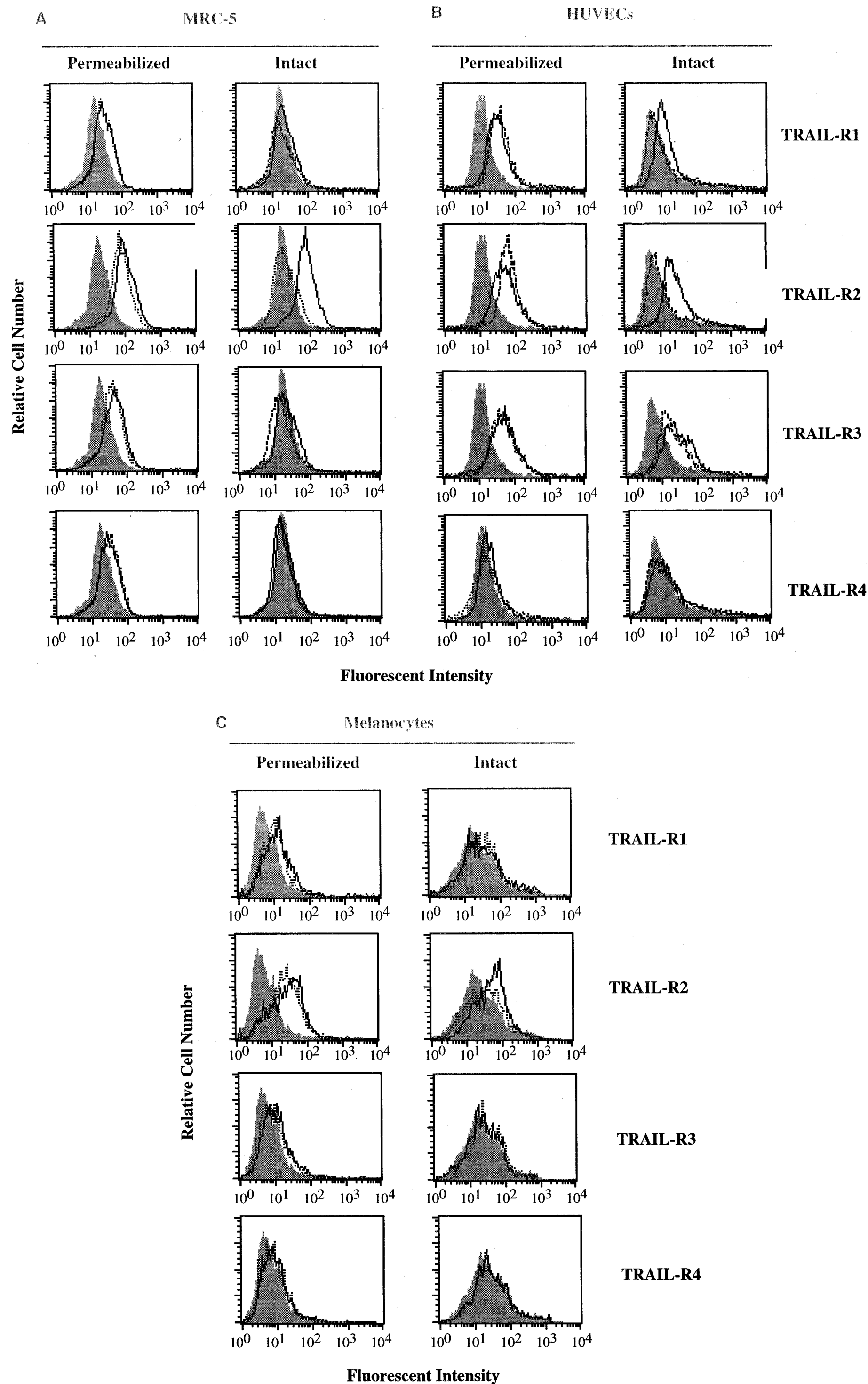

Fig. 1. Flow cytometric analysis of TRAIL-R expression on intact and permeabilised MRC-5 fibroblasts (A), HUVECs (B) and melanocytes (C) without exposure to TRAIL (solid lines) or pretreated with TRAIL (200 ng/ml) at 37°C for 30 min (dotted lines). The filled histograms are isotype controls.

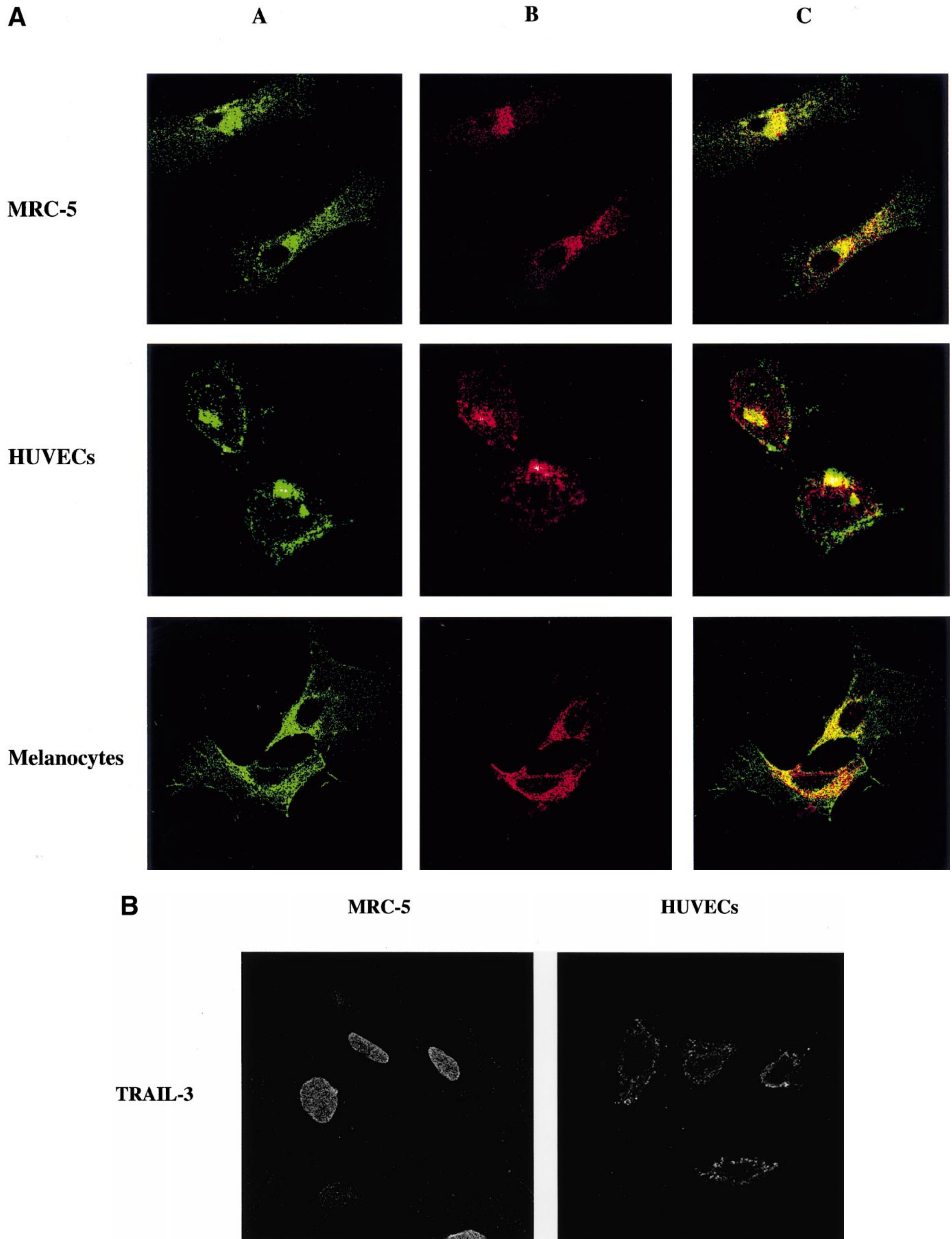

Fig. 2. A: Co-localisation of TRAIL-R2 (A) with the Golgi apparatus (B). The panels under C indicate appearance of merged images. The appearance of TRAIL-R1 in MRC-5 and HUVECs was identical to that of TRAIL-R2. B: The nuclear localisation of TRAIL-R3 in MRC-5 fibroblasts and the cytoplasmic localisation of TRAIL-R3 in HUVECs. The localisation of TRAIL-R4 in MRC-5 is identical to that of TRAIL-R3.

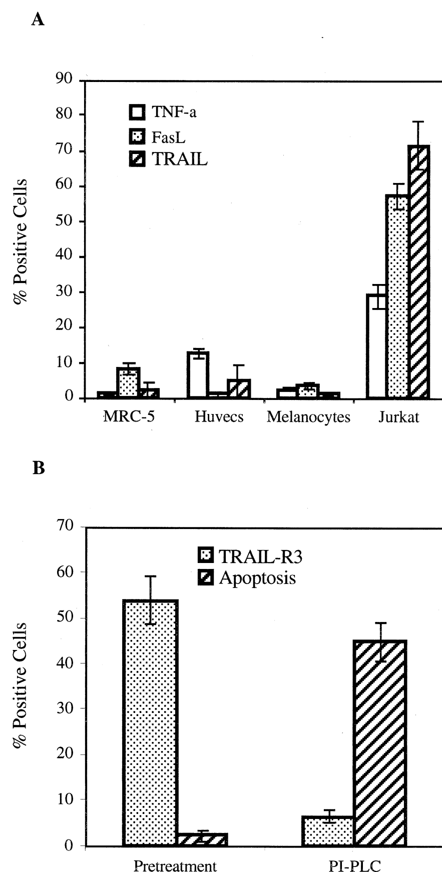

Fig. 3. A: Apoptosis induced by TNF-α, FasL and TRAIL. Cells were treated with TNF-α (100 ng/ml), FasL (1/200) or TRAIL (200 ng/ml) at 37°C for 18 h before measurement of apoptosis. Jurkat T cells were used as a positive control. B: Removal of TRAIL-R3 from the cell surface sensitised HUVECs to TRAIL induced apoptosis. HUVECs treated with PI-PLC (1 μM) at 37°C for 1 h were either subject to flow cytometric analysis for surface TRAIL-R3 expression or exposed to TRAIL (200 ng/ml) in the presence of PI-PLC for 18 h followed by measurement of apoptosis. Data shown were mean ± S.E.M. of three individual experiment.

wheat germ agglutinin (WGA lectin) (Sigma) and the nucleus was identified by propidium iodide (Sigma).

2.2. Immunofluorescence and confocal microscopy

Cells were seeded onto gelatine coated sterile glass coverslips in 24 well plates (Becton Dickinson, N.S.W., Australia) 16–24 h before fixation. Cells were then stained and examined exactly as described elsewhere [20].

2.3. Flow cytometry and measurement of apoptosis

Studies on intact and permeabilised cells were similar to the methods described elsewhere [19,24]. Melanoma cell lines Me10538 and Me4405 were used as the negative and positive control, respectively [19]. Apoptotic cells were determined by the propidium iodide method described elsewhere [25].

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of the cells were prepared as described elsewhere [20]. 3 μg of each extract was incubated with 2 μl of gel shift binding buffer (Promega, N.S.W., Australia) for 10 min at room temperature and then exposed at room temperature for 30 min to double stranded oligonucleotides for the consensus binding sites of NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) that had been labelled with [^{32}P]ATP (Amersham, Buckinghamshire, UK) by polynucleotide kinase (Promega). Protein–oligo complexes were separated on a non-denaturing 6% polyacrylamide gel electrophoresis in 0.5×TBE. Gels were dried and exposed to an X-ray film (Hyperfilm, Amersham).

3. Results

3.1. TRAIL-R expression on MRC-5 fibroblasts, HUVECs and melanocytes

Analysis of TRAIL-R expression on intact and permeabilised cells from cultures of MRC-5 fibroblasts, HUVECs and melanocytes is shown in Fig. 1A–C. MRC-5 fibroblasts expressed TRAIL-R2 but negligible amounts of the other receptors on their surface. Small quantities of the other receptors were however detected within the cells in studies on permeabilised cells. In contrast, HUVECs expressed TRAIL-R1, -R2 and -R3 on the cell surface and within the cells but had negligible expression of TRAIL-R4 on both intact and permeabilised cells. Melanocytes expressed only a small amount of TRAIL-R2 on their surface, whereas higher levels of this receptor and marginal amounts of TRAIL-R1 and -R3 were seen in permeabilised cells. Melanocytes showed no expression of TRAIL-4 on either intact or permeabilised cells.

Fig. 1 also illustrates regulation of surface TRAIL receptor expression after interaction with TRAIL. TRAIL induced marked downregulation of surface expression of death receptors, e.g. TRAIL-R1 on HUVECs and TRAIL-R2 on all three lines. The surface expression of decoy receptors, in particular TRAIL-R3 on HUVECs, remained unaltered. Studies on permeabilised cells suggested that changes induced by TRAIL were due to relocation of the receptors into the cells as total expression in permeabilised cells remained stable. This was confirmed by studies using cycloheximide (CHX, 100 μg/ml) to block protein synthesis and actinomycin D (Act-D, 3 μg/ml) to block transcription in that neither CHX nor Act-D treatment affected changes in expression of TRAIL-Rs (data not shown), which suggested that regulation of TRAIL-R expression by TRAIL is a posttranslational process.

3.2. Subcellular localisation of the receptors by confocal microscopy

Higher expression of TRAIL-Rs in permeabilised cells suggested that intracellular pools of these proteins may exist in normal cells as in melanoma cells [20]. The confocal images in Fig. 2 illustrate the localisation of the TRAIL-Rs. Death receptors, TRAIL-R1 in MRC-5 fibroblasts and HUVECs, and TRAIL-2 in all three cell lines, were predominantly co-localised with the Golgi apparatus that was identified with WGA lectin (Fig. 2A). The localisation of TRAIL-R3 and -R4 varied however between the different cell types. In MRC-5 fibroblasts, staining with mAbs against TRAIL-R3 and -R4 showed co-localisation with the nucleus that was identified with propidium iodide. The expression was diffuse throughout the nucleoplasm (Fig. 2B). In contrast, staining of HUVECs for TRAIL-R3 displayed a sparse punctate cytoplasmic pattern (Fig. 2B). There was no apparent co-localisation of TRAIL-R3 and -R4 with the Golgi apparatus, the endoplasmic reticulum (ER) or the mitochondrion in HUVECs (data not shown). Melanocytes expressed virtually no TRAIL-R3 and -R4 that could be detected by confocal microscopy.

3.3. TRAIL-R3 protects HUVECs from TRAIL induced apoptosis

Apoptosis induced by TRAIL, FasL and TNF-α is shown in Fig. 3A. As expected, none of the normal cells were sensitive to apoptosis induced by these TNF family members. In the case of FasL and TNF-α this could be accounted for by

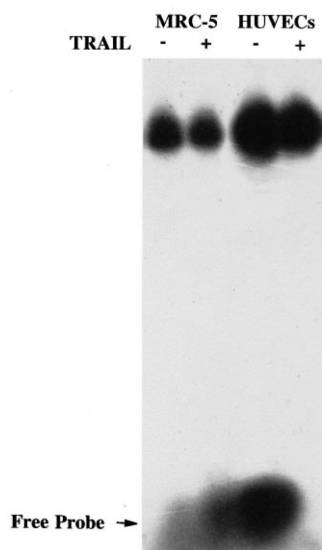

Fig. 4. NF-κB activation status analysed by EMSA. Nuclear extracts from MRC-5 fibroblasts and HUVECs before and after TRAIL treatment (200 ng/ml) at 37°C for 30 min were subject to EMSA.

lack of the corresponding death receptors, Fas and TNFR1, on the cell surface (data not shown). Low surface expression of the death receptors for TRAIL may also be responsible, at least in part, for the resistance of melanocytes to TRAIL induced apoptosis. TRAIL-R3 was shown in previous studies to be a GPI anchored protein on the cell membrane [9]. Pretreatment of HUVECs with recombinant phosphatidylinositol specific phospholipase C (PI-PLC) depleted the cell surface of TRAIL-R3 and sensitised the cells to TRAIL induced apoptosis [9]. A similar experiment is shown in Fig. 3B and confirms the central role that this receptor plays in resistance of HUVECs to TRAIL induced apoptosis. Treatment of MRC-5 fibroblasts and melanocytes with PI-PLC however did not result in an increase in sensitivity of cells to TRAIL induced apoptosis (data not shown).

3.4. *NF-κB is not activated by TRAIL in fibroblasts and HUVECs*

In previous studies on melanoma lines we found that activation of NF-κB appeared to mediate resistance of some melanomas to TRAIL induced apoptosis [21]. To study whether TRAIL induces NF-κB activation in normal cells and thus protects cells from TRAIL induced apoptosis, nuclear extracts from TRAIL treated and non-treated MRC-5 fibroblasts and HUVECs were subjected to EMSA. Fig. 4 shows that treatment with TRAIL did not result in an increase in basal NF-κB activation in MRC-5 fibroblasts or HUVECs, even though TRAIL-R1 and -R2 were expressed on the cell surface of both types of cells (Fig. 1A,B), which are known to be capable of inducing NF-κB activation upon ligation [4,9].

3.5. *Expression of FLICE inhibitory protein (c-FLIP) and inhibitors of apoptosis protein (c-IAP) family members in cultured normal cells*

Intracellular inhibitors of apoptosis c-FLIP and c-IAPs have been suggested to play an important role in resistance of cells to apoptosis. We therefore examined expression of c-FLIP and c-IAP family members, c-IAP1, c-IAP2 and XIAP, in permeabilised MRC-5 fibroblasts, HUVECs and melanocytes. Fig. 5A demonstrates that only a negligible amount of c-FLIP was detected in MRC-5 and HUVECs and none in melanocytes. In contrast, all three lines expressed relatively high levels of all the IAP family members tested (Fig. 5B).

3.6. *Exposure of MRC-5 fibroblasts to TRAIL activates caspase-3*

Activation of caspase-3 is thought to be the 'point of no return' in apoptotic signal transduction [26,27]. We examined whether the failure of TRAIL to induce apoptosis in normal cells takes place upstream of caspase-3 by measuring the activation status of caspase-3 in the cells with a mAb that specif-

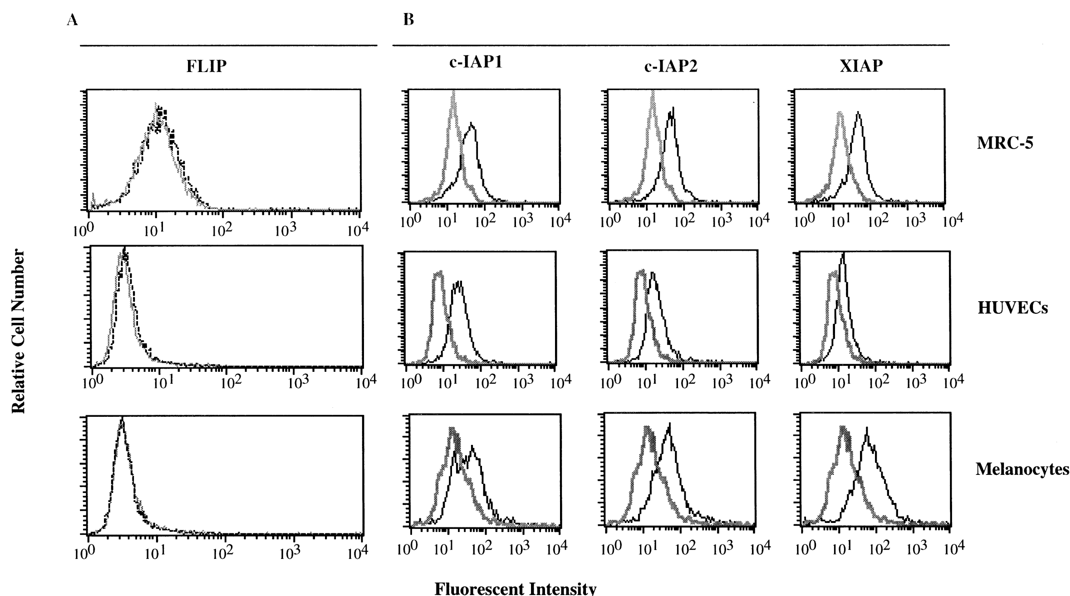

Fig. 5. Flow cytometric analysis of expression (thin lines) of c-FLIP (A) and IAP family members (B) in permeabilised MRC-5 fibroblasts, HUVECs and melanocytes. The thick lines are isotype controls.

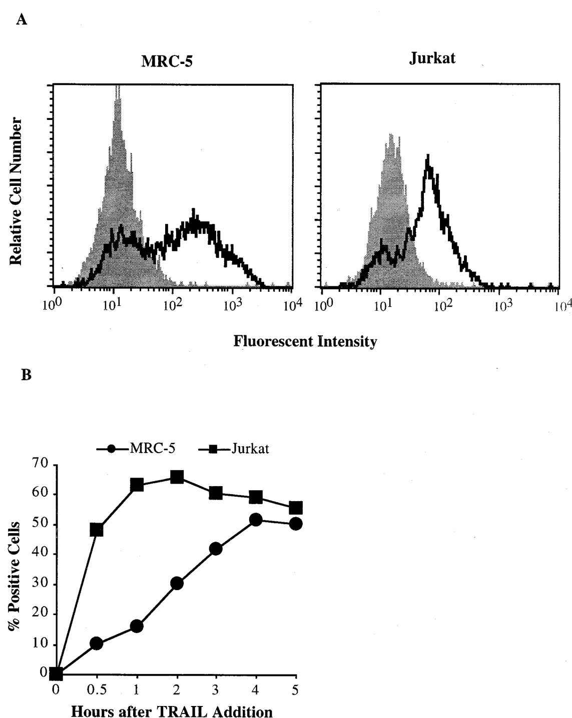

Fig. 6. A: Flow cytometric analysis of activated caspase-3 in MRC-5 fibroblasts and Jurkat T cells treated with TRAIL (200 ng/ml) at 37°C for 4 h before fixation. The filled histograms are isotype controls. B: Kinetics of caspase-3 activation induced by TRAIL. Cells were treated with TRAIL (200 ng/ml) at 37°C for the indicated periods before fixation. Activated caspase-3 was measured by flow cytometry. Data shown are percentage of activated caspase-3 positive cells.

ically recognises the active form of caspase-3. Activated caspase-3 was readily detected in MRC-5 fibroblasts but not in HUVECs and melanocytes upon TRAIL treatment at levels comparable to that seen in the TRAIL sensitive Jurkat T cell line (Fig. 6A). The kinetics of caspase-3 activation in MRC-5 fibroblasts and Jurkat T cells is shown in Fig. 6B and indicates that activated caspase-3 was detectable as early as 30 min after TRAIL treatment in MRC-5 fibroblasts and peaked at 4 h to the level seen in Jurkat T cells.

4. Discussion

The results described above suggest that expression and intracellular distribution of TRAIL-Rs in normal cells vary between cells from different tissues. In HUVECs the two death receptors, TRAIL-R1 and -R2, and the decoy receptor, TRAIL-R3, are expressed on the surface whereas in melanocytes and fibroblasts only the death receptor, TRAIL-R2, was detected on the cell surface. Studies on permeabilised cells show that all receptors except TRAIL-R4 are expressed within the cells to varying degrees. TRAIL-R4 was only weakly detected or absent in the cells. Exposure to TRAIL resulted in marked downregulation of the surface death receptors but had little effect on the decoy receptors in the three types of cells. These results are similar to those found in studies on melanoma cell lines [19,20] and are consistent with PCR analysis of mRNAs for TRAIL-Rs in normal cells [1,3,5].

Co-localisation studies indicated that TRAIL-R1 and -R2 in all three cell lines are predominantly located in the Golgi network and on the cell surface. These results are similar to our previous studies in melanoma cell lines [20] and to studies on TNFR1 and Fas expression [28,29]. Taken together, these findings suggest that localisation in the Golgi apparatus may be a common feature of TNF receptor family members inducing apoptosis. In contrast, the intracellular location of the decoy receptors, TRAIL-R3 and -R4, was found to vary in cells from different tissues. In HUVECs, TRAIL-R3 was expressed in the cytoplasm with a punctate staining pattern, suggesting the location of this receptor in cytoplasmic organelle(s). The organelle(s) involved are not yet known but are not Golgi, ER or mitochondria. In contrast, TRAIL-R3 in MRC-5 fibroblasts was found to be localised in the nucleus. This is consistent with our previous studies on human melanoma cell lines [19,20]. The basis for and significance of the nuclear localisation of TRAIL-R3 is currently unknown, but it is of interest that TRAIL-R3 has sequences compatible with those needed to bind to the nuclear export factor exportin 1. [30,31].

Even though the intracellular location and surface expression of TRAIL-Rs varied between different tissues, all were resistant to TRAIL induced apoptosis. In melanocytes, only a small amount of the death receptor TRAIL-R2 was detected on the cell surface and this could account for their resistance to TRAIL induced apoptosis. This is because we have shown previously that there was a direct correlation between the level of surface death receptor expression and TRAIL induced apoptosis [19].

In the case of HUVECs, TRAIL-R3 expression was clearly needed for resistance to TRAIL induced apoptosis in that, as reported by others, removal of the receptor resulted in sensitivity to TRAIL induced apoptosis [9]. These observations suggest that TRAIL-R3, when expressed on the cell surface at high levels relative to death receptors, may protect cells from TRAIL induced apoptosis. It was notable that TRAIL-R3 in HUVECs was localised in the cytoplasmic organelle(s), whereas TRAIL-R3 in MRC-5 fibroblasts was found to be predominantly localised within the nucleus. This suggests that posttranslational mechanisms that direct the newly synthesised protein to different compartments of the cells may play a critical role in determining whether the decoy receptors may be deployed on the cell surface and thus protect cells from TRAIL induced apoptosis.

MRC-5 fibroblasts expressed high levels of the death receptor TRAIL-R2 but only negligible amounts of the decoy receptor TRAIL-R3 on the surface, yet were not sensitive to TRAIL induced apoptosis. In studies on melanoma cells we found that resistance to TRAIL induced apoptosis was dependent in part on activation of NF-κB [21]. The latter however did not appear to be involved in resistance of normal cells to TRAIL induced apoptosis in that we were unable to show activation of NF-κB by TRAIL in EMSA of MRC-5 fibroblasts and HUVECs.

We also examined whether the cells may contain inhibitors of TRAIL induced apoptosis such as c-FLIP [32,33]. However, the latter was only detected at negligible levels in the cells and was therefore unlikely to account for the resistance to TRAIL induced apoptosis. This is consistent with our previous findings in melanoma cells [19]. The IAP family members c-IAP1, c-IAP2 and XIAP were expressed at high levels in the normal cells and are known to inhibit activation of caspase-9, -3, and -7 [16,17]. It appears unlikely however

that these proteins were responsible for protection against TRAIL induced apoptosis of MRC-5 fibroblasts as activated caspase-3 was detected at high levels in MRC-5 fibroblasts after exposure to TRAIL. It therefore appears that mechanisms downstream of activated caspase-3 were involved in resistance of MRC-5 fibroblasts to TRAIL induced apoptosis.

In summary, our results indicate that TRAIL decoy receptor expression is not the only mechanism involved in protection of normal cells against TRAIL induced apoptosis. Low surface death receptor expression and inhibitory mechanisms downstream of activated caspase-3 appear to be additional mechanisms in protection of melanocytes and fibroblasts respectively.

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