Original Article

Cell-surface Associated p43/Endothelial-monocyte-activating-polypeptide-II in Hepatocellular Carcinoma Cells Induces Apoptosis in T-lymphocytes

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OBJECTIVE: The novel, proinflammatory cytokine endothelial-monocyte-activating-polypeptide-II (EMAP-II) was first found in tumour cell supernatants and is closely related or identical to the p43 component of the mammalian multisynthetase complex. In its secreted form, EMAP-II has multiple cytokine-like activities *in vitro*, including chemotactic, procoagulant and antiangiogenic properties. We recently showed that neoplastic but not normal hepatocytes expresses the 34-kDa molecule on the cell surface *in vitro* and the cell-surface expression is upregulated by treatment with tumour necrosis factor (TNF)-α/interferon (IFN)-γ and/or hypoxia. We hypothesized an immune-regulatory role of EMAP-II within neoplastic tissues and investigated its effects on lymphocytes.

METHODS: To study the role of EMAP-II in tumour cell-induced lymphocyte killing, Jurkat T-cells were co-cultured with a range of hepatocellular carcinoma (HCC) cell monolayers (HuH-7, HepG2 and Alexander cells), which were either untreated or treated with TNF-α/IFN-γ under normoxic and hypoxic conditions over a period of 16–24 hours. Flow cytometric analysis of apoptosis in Jurkat cells was performed using the annexin-V-FITC/propidium iodide technique.

RESULTS: rEMAP-II caused a dose-dependent apoptosis in Jurkat T-cells. Co-culture of Jurkat cells with HCC cell monolayers induced significant apoptosis of the Jurkat cells. In general, under normoxic conditions, cytokine-treated HCC cell monolayer caused more apoptosis than untreated cells. This effect was enhanced by hypoxia. Critically, native EMAP-II expressed on the surface of the HCC cells also induced activation of caspase-8 and apoptosis in Jurkat cells, which was partially but significantly blocked by addition of polyclonal antibodies against EMAP-II to the incubation mixture.

CONCLUSION: Our data suggest that membrane-bound EMAP-II is cytotoxic to lymphocytes and, therefore, might constitute a component of a novel, immunosuppressive pathway by which HCC cells may eliminate attacking T-cells and evade the immune system. The mechanism by which it does so is currently under investigation. [*Asian J Surg* 2007;30(1):13–22]

Key Words: apoptosis, EMAP-II, hepatocellular carcinoma

Introduction

Endothelial-monocyte-activating-polypeptide-II (EMAP-II) was initially isolated from the supernatant of cultured murine tumour cells[1] and was so named because of its pleiotrophic activities towards endothelial cells, monocytes/macrophages and neutrophils. The full functions of EMAP-II in both normal and malignant tissues have yet to be

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determined, but it clearly has important roles in the mediation of inflammatory response and coagulation. Purified EMAP-II protein induces expression of E- and P-selectin on endothelial cells *in vitro*, release of von-Willebrand factor, chemotaxis of neutrophils and macrophages, and activation of neutrophil respiratory burst.[2] *In vivo*, injection of EMAP-II stimulates an inflammatory infiltrate in the mouse footpad and some regression of implanted tumours.[2,3] EMAP-II has also been implicated in lung morphogenesis,[4] and has been shown to induce endothelial cell apoptosis and inhibit angiogenesis.[5] On the basis of these findings, EMAP-II has been described as a proinflammatory cytokine with anti-angiogenic activity.

Full-length complementary deoxyribonucleic acids (cDNAs) encoding human EMAP-II have been isolated[2,6] and is consistent with a 34-kDa molecule of 328 amino acid residues, which is proteolytically cleaved at a critical aspartate residue (Asp^{144}) to produce a smaller ~20-kDa polypeptide. One study shows that the 34-kDa molecule is sensitive to cleavage by caspase-7,[7] while other studies suggest that other proteases may be more important.[8,9] The p43 auxiliary component of the mammalian multisynthetase complex shares 86% and 85% amino acid identity with human and murine 34-kDa EMAP-II, respectively, and the human p43 and EMAP-II are identical.[10,11] It has been suggested that a wide range of cultured cells, normal and transformed, retain the 34-kDa form of p43/EMAP-II in the cytoplasm while only some tumour cell lines release fully or partially processed smaller forms of EMAP-II into the culture medium, either constitutively, as a result of cellular stress,[12,13] or as a consequence of activation of programmed cell death.[14] This soluble form of EMAP-II produced by some colorectal cancer cells has been shown to induce apoptosis in activated lymphocytes.[11,15]

A recent immunohistochemical study of normal human tissues suggested that tissues with high level of protein synthesis and/or endocrine function are rich in EMAP-II.[11] Liver being an important organ for such functions, we hypothesized that malignant transformation of hepatocytes might cause overexpression of EMAP-II and studied the regulation of expression of this molecule in hepatocellular carcinoma (HCC) cells, as it has not been looked for before. We then sought a biological rationale for the expression of EMAP-II by the malignant hepatocytes, other than acting as a chemoattractant for phagocytic cells. If inflammatory cells were attracted into the tumour microenvironment to clear cellular debris, a mechanism to simultaneously inhibit the activity of tumour-specific T-cells would be advantageous from a tumour perspective. We hypothesized that EMAP-II might therefore be cytotoxic towards lymphocytes and tested this using cell culture models of tumour–lymphocyte interaction.

Materials and methods

Cell lines and characterization of cells

The human leukaemic T-cell line Jurkat (clone E6.1), colorectal adenocarcinoma cell line DLD-1, a normal hepatocyte cell line THLE-3 and HCC cell lines PLC/Prf/5 (Alexander cells) and HepG2 cells were all obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and HuH-7 from Japanese Collection of Research Bioresources (JCRB, Japan). All cell culture reagents and additives were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. Flasks used to propagate THLE-3 cells were coated (2 hours at 37°C) with 0.01 mg/mL bovine serum albumin, 0.01 mg/mL fibronectin and 0.03 mg/mL collagen I. THLE-3 cells were cultured in William's media E supplemented with bovine pituitary extract 50 μg/mL, hydrocortisone 0.5 μg/mL, hEGF 0.5 ng/mL, transferrin 10 μg/mL, insulin 5 μg/mL, retinoic acid 1×10^{-4} ng/mL, tri-iodothyronine 6.5×10^{-3} ng/mL and 70 ng/mL phosphoethanolamine. 100 U/mL penicillin–streptomycin solution, 4 nM L-glutamine and 10% heat inactivated fetal calf serum (FCS) were also added to the basal media. DLD-1 cells were cultured in RPMI 1640 media supplemented with 10% FCS and 100 U/mL penicillin–streptomycin. Alexander cells, HuH-7, HepG2 and Jurkat cells were all grown in Dulbecco's minimum essential medium (DMEM) with 10% FCS, 4 nM L-glutamine and 100 U/mL penicillin–streptomycin. All cells were routinely grown in corning-incorporated T-75 tissue culture flasks (Corning Inc., NY, USA) and cultured in a humidified incubator at 37°C, with 5% CO_2, and routinely subcultured by removal from flasks with 0.05% trypsin/1 mM ethylenediamine tetraacetic acid.

THLE-3, Alexander cells, HuH-7 and HepG2 were all characterized for phenotypic characteristics of hepatocytes using expression of albumin, alpha-fetoprotein (AFP),[16] cytokeratin-18[17] as positive markers by reverse transcriptase–polymerase chain reaction, Western blotting and fluorescent microscopy and uptake of indocyanine green (ICG) by spectrophotometric analysis.[18] All cells strongly expressed albumin and cytokeratin-18 and showed high absorbance

for ICG uptake at 800 nm; all cells except THLE-3 also expressed AFP (data not shown).

Antibodies (Abs) and recombinant proteins

Rabbit polyclonal Abs against EMAP-II (Bio-Source Intl., CA, USA) or in-house (R2B2) were used for immunohistochemistry, Western blotting and flow cytometry in this study. The characteristics of the Abs, which recognize the 34- and 20-kDa, as well as several intermediate forms of EMAP-II, have been described elsewhere.[7,9,13] Horseradish peroxidase (HRP)- and fluorescein isothiocyanate (FITC)-labelled anti-rabbit IgG Abs (DAKO, Glostrup, Denmark) were used for detection of primary Abs. Recombinant human tumour necrosis factor (TNF)-α and interferon (IFN)-γ were obtained from PeproTech (London, UK).

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed, paraffin-embedded specimens of normal and HCC affected human liver tissue for EMAP-II expression. Slides were dewaxed in histolene, before being rehydrated in graded ethanol solutions (100% to 30%). Antigen retrieval was performed by boiling the slides for 10 minutes in citrate buffer (10 mM citric acid, 25 mM NaOH). Slides were blocked with normal goat serum for 20 minutes. EMAP-II was identified by incubating the slides with purified polyclonal anti-EMAP-II Abs (1 μg/mL in phosphate buffered saline [PBS]) for 1 hour at room temperature. Secondary detection was performed using Vectastain Elite Kit, according to the manufacturer's instruction (Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with haematoxylin solution (Vector Laboratories), dehydrated in ethanol, and mounted with depex polystyrene solution (BDH, Poole, UK).

Detection of EMAP-II by Western blotting

Western blotting was conducted essentially as previously described.[7] Briefly, cells were trypsinized and washed with cold PBS, counted and 5×10^6 cells were lysed in 400 μL sodium dodecyl sulfate (SDS) buffer (0.5 M Tris-HCl, 10% glycerol, 10% w/v SDS, 2% 2-ME, 0.1% bromophenol blue). Supernatant proteins were precipitated with ice-cold acetone and resuspended in 400 μL SDS buffer. Samples were boiled for 10 minutes, total protein assay of the samples was performed using Bio-Rad (Bio-Rad, CA, USA) reagents and protocol and equal amount of total protein of different samples was loaded for electrophoresis by SDS-PAGE (polyacrylamide gel electrophoresis) on 10% gels, and transferred onto nitrocellulose membranes (Amersham, Bucks, UK). Membranes were blocked overnight with 5% non-fat dried milk in 0.5% Tween 20/PBS, and exposed to anti-EMAP-II Abs, diluted in blocking solution for 2 hours at room temperature. Proteins were visualized with HRP-conjugated goat anti-rabbit IgG using the ECL system (Amersham).

Flow cytometric analysis of cell surface expression of EMAP-II

The expression of EMAP-II on the external membranes of hepatocytes was determined by flow cytometry. Cells were incubated in DMEM with or without 20 ng/mL TNF-α/IFN-γ in normoxia or hypoxia for 24 hours. After removal of the medium, cells were harvested without trypsin to reduce membrane damage, and fixed in 0.25% glutaraldehyde/PBS for 10 minutes to restrict access of Abs to cell surface antigens only. Cells were washed in PBS and incubated with anti-EMAP-II polyclonal antibody (R2B2) 1 μg/mL for 2 hours. Surface EMAP-II expression was detected by subsequent incubation with FITC-conjugated secondary Abs (Sigma-Aldrich) for 30 minutes. Additional cell samples were incubated with the secondary FITC-conjugated Ab, but without primary Ab, as controls. The cells were immediately analysed by flow cytometry. A total of 20,000 events were stored for each sample.

Assessment of apoptosis by FITC-labelled annexin-V and propidium iodide (PI)

Annexin-V binds to phosphatidylserine (PS) exposed on the external surface of the plasma membrane during the early phase of the apoptotic programme, while DNA within the nucleus only becomes accessible to PI during late apoptosis. Healthy living cells should therefore bind to neither of these dyes, and these two reagents can be used together to distinguish early and late apoptosis. The POPTEST™-FITC (DAKO) employs the property of annexin V-FITC to bind to PS in the presence of Ca^{2+}. Jurkat cells, following treatment with either tumour-conditioned media, recombinant EMAP-II or after co-culture with hepatocyte monolayers, were assessed for apoptosis. Untreated control cells and cells treated with 20 ng/mL of TNF-α/IFN-γ were also prepared. Samples were analysed in accordance with the manufacturer's protocol. Briefly, the cells of interest (i.e. Jurkat cells) were washed with ice-cold culture medium and finally suspended in ice-cold diluted binding buffer at

10^5–10^6 cells/mL. One microlitre of annexin V-FITC and 2.5 μL of PI were added to each cell sample and left on ice in the dark for 10 minutes. The samples were immediately analysed using a Becton Dickenson FACScan, using the LYSIS II programme. Analysis was restricted to the lymphocyte population by gating on forward and right angle scatter. A total of 10,000 events were acquired for each analysis.

Caspase-8 assay

A colorimetric assay based on the specific cleavage of the peptide substrate Ile-Glu-Thr-Asp-*p*-nitroanilide (IETD-pNA) by active caspase-8, releasing pNA, was used as directed by the manufacturer (Bio-Source). Active caspase-8 activity was measured in extracts of control Jurkat cells, cells co-cultured over a period of 12–24 hours with hepatocytes untreated or treated with 20 ng/mL of TNF-α/IFN-γ in the presence or absence or anti-EMAP-II Abs.

Conditioned medium and co-culture experiments

All adherent cell monolayers (THLE-3, DLD-1, Alexander, HepG2 and HuH-7) were grown for 48 hours in serum-free medium, in medium supplemented with TNF-α/IFN-γ (20 ng/mL each), or in medium under hypoxic conditions (~1–2% O_2). Used culture medium was centrifuged at 2,000*g* for 5 minutes to remove cellular debris and stored at −80°C. Jurkat cells were pelleted, resuspended in conditioned medium and grown in normoxia or hypoxia for 48 hours before analysing for apoptosis by the annexin-V/PI method, as described previously. In co-culture experiments, cells were grown up to 50–60% confluence. Cell monolayers were then treated with TNF-α/IFN-γ (20 ng/mL each) for 24 hours in normoxia or hypoxia. The media were then removed, cell monolayer washed with PBS and Jurkat cells (4×10^6) were co-cultured with the adherent monolayer for 24 hours. Before addition of Jurkat cells, some monolayers were treated with 10 μg/mL of anti-EMAP-II Ab for 1 hour. Functional blocking of EMAP-II with this concentration of R2B2 antibody has previously been demonstrated.[6] After 24 hours, the Jurkat cells were removed and centrifuged at 500*g* for 5 minutes, and then resuspended in PBS. Samples were analysed using the annexin-V/PI technique described previously.

Statistical analysis

Analysis of FACScan data was performed using WinMDI software 2.8 (TSRI Cytometry, La Jolla, CA, USA). Quantitative analysis of positive immunohistochemical staining for EMAP-II was done using the Simple PCI image analysis software (version 5.1.0.0110, Cranberry Township PA, USA). Student's *t* test and one-way analysis of variance was used where appropriate for testing the significance of the data. All statistical tests were performed using SPSS (SPSS Inc., Chicago, IL, USA) and a p value of ≤ 0.05 was considered significant. All experiments were performed in triplicates and data shown are mean ± standard deviation.

Results

Expression of EMAP-II in normal and malignant human liver in vivo

Initially, we studied the expression of EMAP-II in archival specimens of normal and HCC liver samples by immunohistochemistry. In general, hepatocytes in tissue sections showed strong cytoplasmic immunoreactivity for EMAP-II with a punctate pattern (Figure 1A), while the Kupffer cells and bile ductules were generally negative. EMAP-II staining appeared generally more intense within the hepatocytes closer to the central vein, an observation that was subsequently confirmed by image analysis. There was significant gradual increase in expression of the polypeptide from zone 1 to 3 of hepatic acini ($p < 0.005$) (Figure 1B). This pattern of expression was lost in HCC, where the malignant parenchymal cells showed an increased, widespread and generalized staining, while the stromal cells were still negative (Figure 1C).

Expression of EMAP-II in normal and malignant hepatocytes in vitro

We then examined expression of EMAP-II in a range of cell lines derived from HCC and normal liver, to determine whether expression persisted *in vitro*, and whether these cells retained EMAP-II within the cell, or released it into the culture medium. Figure 2 shows a Western blot of extracts from DLD-1 (colorectal carcinoma), THLE-3, Alexander and HuH-7 cells and supernatant. All the cell lines expressed EMAP-II within the cell, predominantly in the 34-kDa form (*lanes 1, 3, 5 and 7*). In contrast to colorectal carcinoma conditioned medium that contained detectable levels of soluble EMAP-II (*lane 2*) none of the hepatocyte cell lines secreted any EMAP-II in the culture medium (*lanes 4, 6 and 8*).

The expression of EMAP-II on the surface of membrane-stabilized hepatocyte cell lines under normoxic and hypoxic conditions was then studied by flow cytometry using

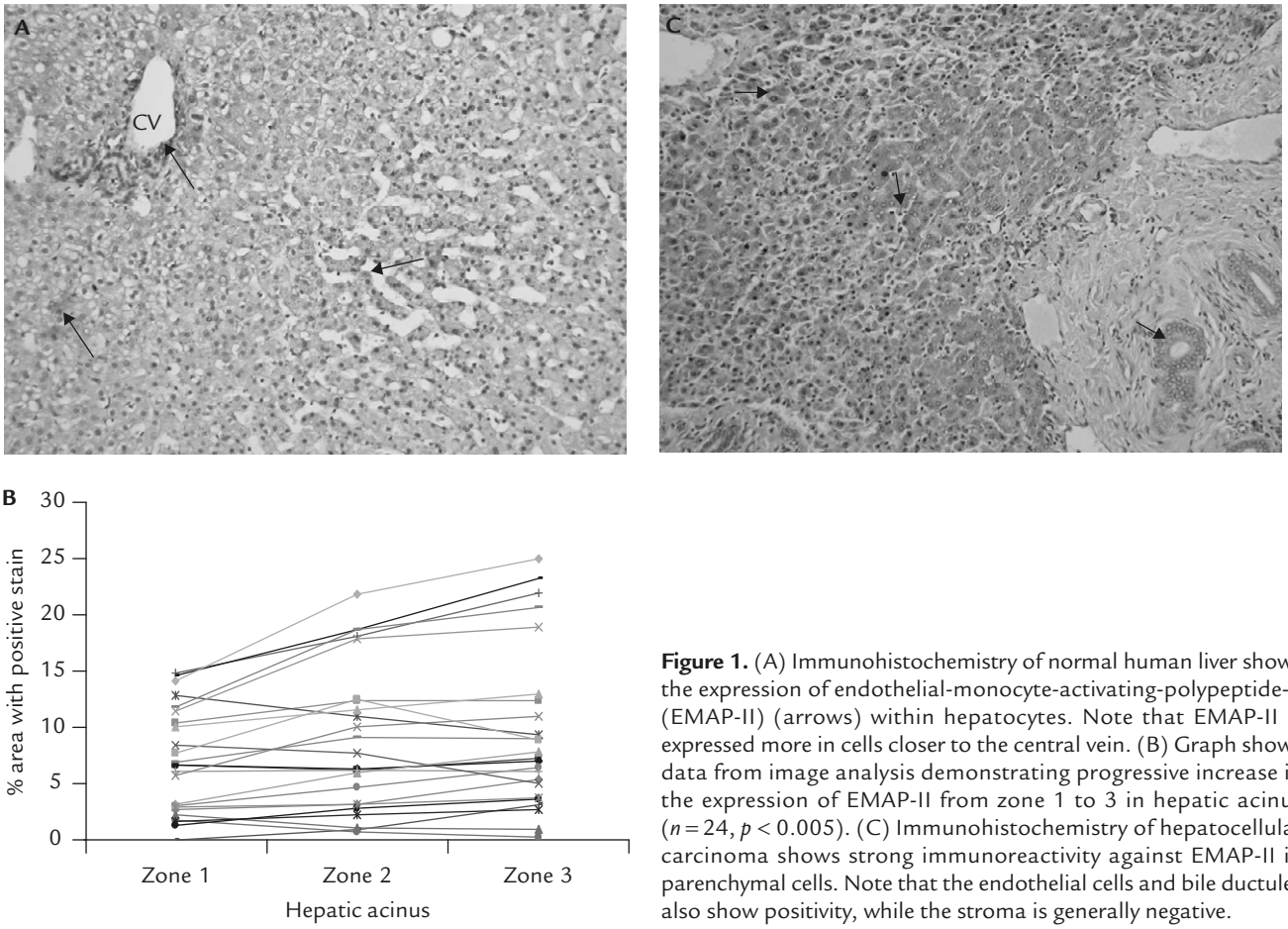

Figure 1. (A) Immunohistochemistry of normal human liver shows the expression of endothelial-monocyte-activating-polypeptide-II (EMAP-II) (arrows) within hepatocytes. Note that EMAP-II is expressed more in cells closer to the central vein. (B) Graph shows data from image analysis demonstrating progressive increase in the expression of EMAP-II from zone 1 to 3 in hepatic acinus ($n = 24$, $p < 0.005$). (C) Immunohistochemistry of hepatocellular carcinoma shows strong immunoreactivity against EMAP-II in parenchymal cells. Note that the endothelial cells and bile ductules also show positivity, while the stroma is generally negative.

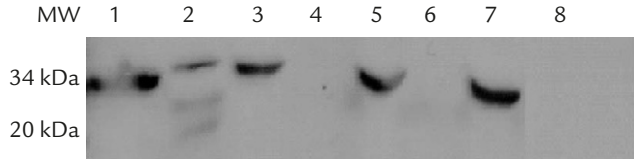

Figure 2. Colorectal carcinoma cells, hepatocellular carcinoma and normal hepatocytes express endothelial-monocyte-activating-polypeptide-II (EMAP-II) *in vitro*. Western blot of extracts of different cells and supernatant, using anti-EMAP-II antibody. *Lane 1* = DLD-1 cell extract; *lane 2* = DLD-1 supernatant; *lane 3* = THLE-3 cell extract; *lane 4* = THLE-3 supernatant; *lane 5* = Alexander cell extract; *lane 6* = Alexander supernatant; *lane 7* = HuH-7 cell extract; *lane 8* = HuH-7 supernatant.

R2B2 polyclonal Abs (Figure 3). THLE-3 cells did not express any EMAP-II on the cell surface under normoxic or hypoxic conditions. A small proportion of Alexander cells expressed surface EMAP-II when grown under normoxic conditions but demonstrated a four- to fivefold increase in surface EMAP-II when exposed to hypoxia. HuH-7 cells expressed EMAP-II on the cell surface under normal condition and showed about twofold increase in surface expression under hypoxic conditions. Treatment with the combination of TNF-α/IFN-γ caused a further two- to threefold increase in mean fluorescence (data not shown).

Cell surface-associated EMAP-II induces apoptosis in Jurkat cells

As demonstrated above, malignant but not normal hepatocytes express EMAP-II on the cell surface, although they do not release this protein into the medium. Because the co-culture model does not require previously conditioned medium, the hepatocytes can be exposed to cytokines to stimulate EMAP-II expression, and the medium exchanged before co-culturing with Jurkat cells. Therefore, at no time are the target Jurkat cells exposed to exogenous cytokines, and the subsequent killing of Jurkats may be attributed to the inductive influence of the cytokines on the hepatocytes. Figure 4 shows a representative flow cytometric analysis of apoptosis in target Jurkat cells following 16-hour co-culture with untreated or treated (with TNF-α/IFN-γ) Alexander and HuH-7 cells. As expected, both the cell lines induced apoptosis in Jurkat cells and the treated

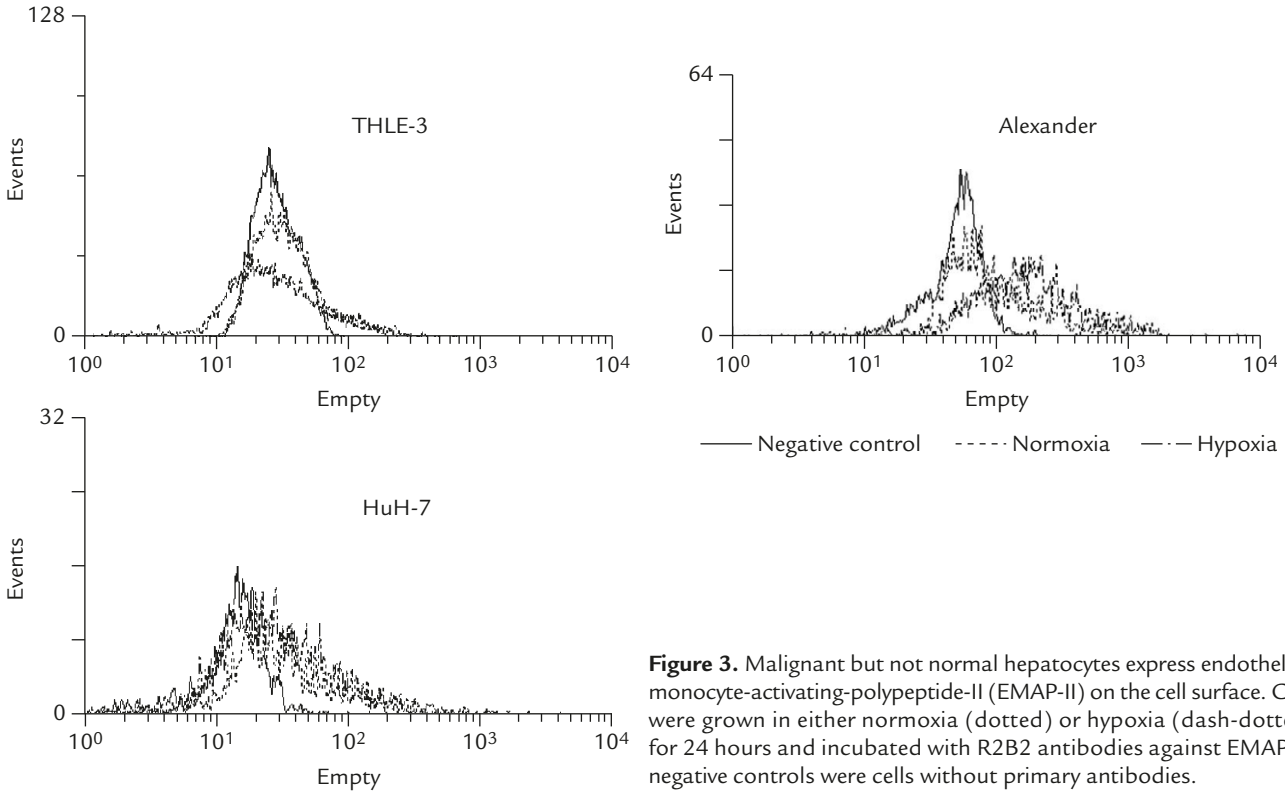

Figure 3. Malignant but not normal hepatocytes express endothelial-monocyte-activating-polypeptide-II (EMAP-II) on the cell surface. Cells were grown in either normoxia (dotted) or hypoxia (dash-dotted) for 24 hours and incubated with R2B2 antibodies against EMAP-II; negative controls were cells without primary antibodies.

cells induced more cell death in Jurkats than untreated ones. Figure 5 shows a graph from pooled data indicating that malignant hepatocytes induce significant apoptosis in Jurkat cells compared to media alone or normal hepatocytes. However, none of the HCC cell-conditioned media induce any significant apoptosis in Jurkat cells compared to the controls (data not shown).

To demonstrate that at least part of the apoptosis induced in Jurkat cells by the hepatocytes is attributable to EMAP-II, co-culture experiments were carried out with or without blocking concentrations of anti-EMAP-II Abs. As shown in Figure 6, there was significant inhibition of apoptosis in Jurkat cells by HepG2 cells when co-cultured with anti-EMAP-II Ab, while normal hepatocytes did not induce any apoptosis by EMAP-II dependent manner.

Activation of caspase-8 in hepatocyte-induced apoptosis of Jurkat cells

Caspase-8 is a member of interleukin-1β (IL-1β) converting enzyme family of cysteine proteases (caspases). It is the most upstream caspase in the CD95 apoptotic pathway. Caspase-8 provides a direct link between cell-death receptors and caspases. Uninduced Jurkat cells and cells co-cultured with THLE-3 showed very low activity of caspase-8, while cells co-cultured with all three malignant hepatocyte cell-lines showed four- to sixfold increase in caspase-8 activity over a period of 12–24 hours (Figure 7).

Effect of hypoxia on expression of cell-surface EMAP-II and its implication on Jurkat cell apoptosis

Under normoxic conditions, the malignant but not the normal hepatocytes expressed EMAP-II on the cell surface. The surface expression was upregulated by exposure of the cells to hypoxia over a period of 16–24 hours (Figure 3). But our Western blot analysis of cell extracts from cells grown under different conditions failed to show any true upregulation of the protein (data not shown), which implies that hypoxia causes an increased membrane translocation of EMAP-II in malignant cells rather than increases protein synthesis *per se*. Subsequently, when cells grown under hypoxia were co-cultured with Jurkat cells, they induced more apoptosis than cells grown under normoxia in an EMAP-II dependent manner, as expected (Figure 4E/F).

Discussion

Tumour cells must develop strategies to avoid clearance by the immune system to survive, expand their populations and metastasize. The process by which unwanted

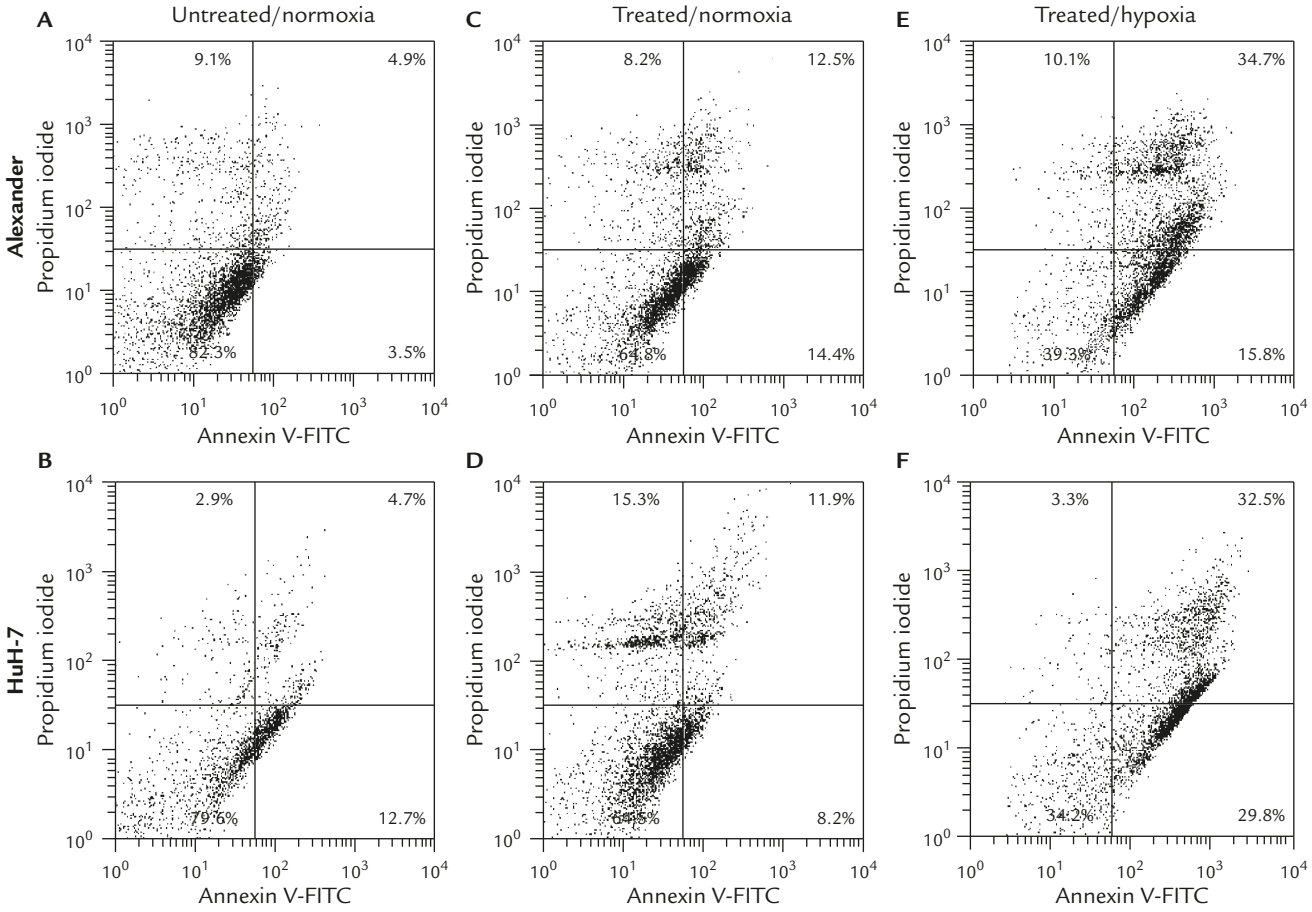

Figure 4. Cell surface-associated endothelial-monocyte-activating-polypeptide-II induces apoptosis in Jurkat cells. (A, B) Cells were co-cultured with either untreated Alexander or HuH-7 cells in normoxia; (C, D) tumour necrosis factor-α/interferon-γ treated cells in normoxia or (E, F) treated cells in hypoxia and analysed by flow cytometry using the annexin-V/propidium iodide technique. Cells in the right lower and upper quadrants are early and late apoptotic, respectively.

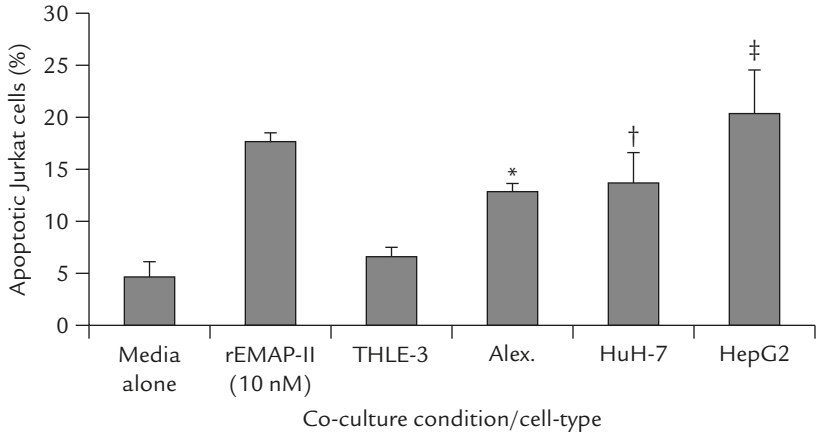

Figure 5. Hepatocellular carcinoma (HCC) cells but not normal hepatocytes induce apoptosis in Jurkat cells in co-culture. Jurkat cells were co-cultured with normal hepatocytes or HCC cells or in media alone for 24 hours and then assessed for apoptosis using flow cytometry. *$p = 0.1$; †$p = 0.03$; ‡$p = 0.005$.

tumour cells are cleared involves recognition of the altered nature of the cell by the immune system followed by its effective killing and elimination. Thus, tumour cells may escape immune clearance by a diversity of mechanisms including altering immune recognition or by modulation of the cytotoxic response. The recent identification of the Fas receptor (Fas, APO-1/CD95) and its ligand (FasL, CD95L) as a major regulator of both apoptosis and immune

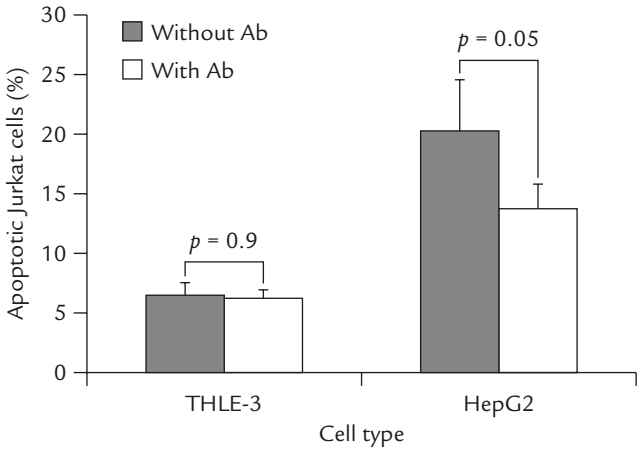

Figure 6. Significant inhibition of apoptosis of Jurkat cells by malignant but not normal hepatocytes when co-cultured with anti-endothelial-monocyte-activating-polypeptide-II antibody, as assessed by the annexin-V/propidium iodide method.

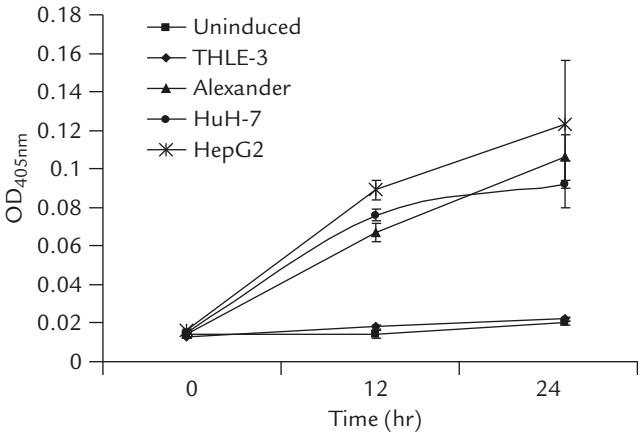

Figure 7. Caspase-8 is activated in Jurkat cells co-cultured with hepatocellular carcinoma (HCC) cells, but not in cells cultured with normal hepatocytes. Caspase-8 activity is measured in uninduced Jurkat cells and cells co-cultured with normal hepatocytes and HCC cells using a colorimetric method. OD = optical density.

function has provided insight into a range of attractive mechanisms by which tumours escape from immune clearance.[19] In HCC, partial or complete loss of Fas expression has been detected, and this loss is supposed to result in reduced sensitivity of the tumour cells towards T-cell toxicity.[20,21] Furthermore, HCC cells have been shown to generate soluble Fas (sFas) to antagonize FasL killing by activated lymphocytes.[21] Here, we report a novel mechanism employed by HCC cells to evade the immune response directed towards them.

Our initial experiments demonstrated that normal human liver expresses EMAP-II within the cytoplasm *in vivo*. The compartmentalized pattern of distribution of EMAP-II in hepatic acini may be an extension of the physiological property of the liver, which sequesters more enzymes and cytokines in zone 3 than zone 1, or might be a response to differing levels of oxygen tension in different zones. However, this pattern of expression was not seen in HCC, which showed an increased expression of EMAP-II in a more generalized and widespread fashion.

Subsequently, we demonstrated by Western blotting that the EMAP-II expression persisted *in vitro* in both normal and malignant hepatocytes. This is consistent with our hypothesis that all human cells, both normal and malignant, express the 34-kDa form of EMAP-II intracellularly as part of multisynthetase complex in the form of p43, but is only processed and released under certain conditions, malignant transformation being one of them. Unlike some colorectal cell lines, which secrete soluble form of EMAP-II in the culture medium, HCC cells did not produce any detectable soluble forms of EMAP-II. Rather, they expressed EMAP-II on the cell surface, which was further upregulated by treatment with TNF-α/IFN-γ and/or hypoxia. Since hypoxia or regions of low oxygen tension is a physiological component of the tumour microenvironment, this observation strongly suggests the possibility of increased EMAP-II expression by malignant cells. As Western blots of washed cell extracts only demonstrated the 34-kDa form, it would appear that this is likely to be the major form associated with the external membrane.

The immunohistochemistry result in conjunction with the flow cytometric data showing increased surface upregulation of EMAP-II in HCC cells under different stimulation implies that the increased surface expression of EMAP-II does not result from an increased synthesis of EMAP-II *per se*, but is rather a membrane translocation phenomenon, which would explain why we could not detect any increased EMAP-II in HCC cells by Western blotting.

The cell surface expression of EMAP-II subsequently induced significant apoptosis in activated lymphocytes, which could be significantly inhibited by anti-EMAP-II Abs, as measured by the annexin-V/PI assay. Normal hepatocytes, which did not express surface EMAP-II, were not toxic to Jurkat cells, confirming our initial hypothesis.

This is the first demonstration of biological activity of cell surface-associated EMAP-II in HCC cells, about which very little is known. The mechanism by which EMAP-II is translocated from inside the cell to the cell membrane is also unknown. The EMAP-II precursor lacks a hydrophobic

signal peptide necessary for membrane translocation, nor is it predicted to be glycosyl phosphatidylinositol anchored. It has been suggested that the mature protein is secreted via a novel pathway, in a similar manner to the leaderless precursor of IL-1β, which is now known to be shed initially in the form of membrane microvesicles.[22] Our data suggest that besides the soluble form of EMAP-II, surface expression alone and subsequent cell–cell contact may be sufficient to render EMAP-II active against lymphocytes.

The mechanism by which EMAP-II induces apoptosis is poorly understood; however, our data, demonstrating activation of caspase-8 in Jurkat cells following co-culture with HCC cells, strongly implicate a death receptor pathway in apoptosis initiated by EMAP-II. Berger et al[5] have shown that EMAP-II upregulates the expression of TNF-R1 (p55) on endothelial cells *in vitro*. TNF-R1 and Fas, both members of the TNF receptor family, are believed to be primary receptors for the initiation of death signals in lymphocytes.[23] The Jurkat cell clone (E6.1) used in our experiments expresses very little FasL on the surface. Furthermore, we recently demonstrated activation of phospho-p38 in this clone of Jurkat cells treated with recombinant EMAP-II, which suggests that EMAP-II might be involved in the initiation of apoptosis via the TNF-R1 mediated pathway (Symonds et al, unpublished data). Therefore, this protein could be acting directly through death receptor, which would group it with TNF, FasL and TNF-related apoptosis-inducing ligands, or it could be inducing or potentiating one of these death ligand/receptor interactions.

Involvement of EMAP-II in viral brain disease in rats with Borna disease virus has been evaluated and a significant positive correlation between EMAP-II expression in ramified microglial cells and viral load in different brain regions has been demonstrated.[24] In light of the fact that HCC develops on top of chronic liver disease and viral hepatitis, it would be interesting to study if viral hepatitis and chronic liver disease cause an overexpression of EMAP-II in the liver and whether it has any implications in chronic viral subversion of the immune system leading to eventual hepatocellular carcinogenesis.

The emerging experimental and clinicopathological evidence indicates an important role of defensive strategies aimed at avoiding immune-detection by tumour-infiltrating lymphocytes as a means of immune escape in hepatocellular cancer. Although the mechanism of EMAP-II-induced apoptosis remains unclear, it is apparent that EMAP-II constitutes a novel component by which tumour cells eliminate attacking T-cells and evade the immune system. Translating this knowledge to clinical practice may eventually prove valuable in developing rational immune-based therapies for HCC.

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