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Functional expression of TRAIL receptors TRAIL-R1 and TRAIL-R2 in esophageal adenocarcinoma

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ABSTRACT

The tumour necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the TNF superfamily that preferentially induces apoptosis in cancer cells, while sparing normal cells. TRAIL induces apoptosis by interacting with its receptors TRAIL-R1 and TRAIL-R2. Recently, new humanized agonistic anti-TRAIL-R1 and anti-TRAIL-R2 antibodies have been developed, and are undergoing phase I/II clinical trails. Esophageal adenocarcinoma (EA) is associated with significantly poor outcome and is rapidly increasing in incidence in the United States and Western Europe, with virtually no effective non-surgical treatment. The aim of this study was to determine whether human EA tissue express TRAIL-R1 and/or TRAIL-R2, and whether EA cell lines Bic-1 and Seg-1 expresses functional TRAIL-R1 and/or TRAIL-R2. The expression of TRAIL-R1 and TRAIL-R2 was determined in sections from 18 human EA by immunohistochemistry (IHC). Sixteen (89%) of the EA expressed TRAIL-R1 and 17 (94%) expressed TRAIL-R2. Both cell lines were found to express TRAIL-R1 and TRAIL-R2 by western blot analysis, IHC, and flow cytometry. The fully human agonistic TRAIL-R1 (HGS-ETR1) and TRAIL-R2 (HGS-ETR2) antibodies induced apoptosis in Bic-1 and Seg-1 cells in a time and dose dependent manner. Our results show that the vast majority of primary human EA express TRAIL-R1 and TRAIL-R2 and that EA cells lines express functional TRAIL-R1 and TRAIL-R2. Targeting of these receptors by agonist monoclonal antibodies may be of therapeutic value in patients with EA.

1. Introduction

The incidence of esophageal adenocarcinoma (EA) has been rising in the United States since the 1970s.[1] Surgery is the only effective treatment for this cancer which is associated with a 3 year survival rate of only 20%,[1] although recent studies show that neoadjuvant chemoradiation may be of benefit in advanced cancer.[2]

The tumour necrosis factor (TNF) related apoptosis-inducing ligand TRAIL/Apo2L belongs to the TNF superfamily.[3,4] TRAIL has four receptors: TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), and TRAIL-R4 DcR2.[5–7] TRAIL-R1 and TRAIL-R2 are death receptors, whereas TRAIL-R3 and TRAIL-R4 are decoy receptors.[7,8] TRAIL receptors were found to be expressed in tumour, transformed, and normal cells.[9,10] TRAIL induces apoptosis by activating its two death

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receptors (TRAIL-R1 and TRAIL-R2) with subsequent activation of the extrinsic and the intrinsic apoptotic pathways.[5,11] Several mechanisms of resistance to death receptor activation by TRAIL have been proposed in different cell types.[5,12–17] Many of these resistance mechanisms have been described for TRAIL, which binds to all of its four receptors. Whether any of these resistance mechanisms apply to agonistic anti-TRAIL death receptor antibodies that selectively trigger only one TRAIL death receptor, remains unknown. Different formulations of soluble TRAIL have demonstrated activity against a wide variety of haematological malignancies, some of which are currently being considered for clinical development.[7,18,19]

Recently, selective agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 has been developed.[20,21] The aim of this study was to determine whether EA expresses functional TRAIL-R1 and TRAIL-R2, to provide alternative treatment strategies by targeting these receptors.

2. Materials and methods

The research protocol was approved by the Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals. The human esophageal adenocarcinoma (EA) cell lines Bic-1 and Seg-1 were obtained from Dr. David G. Beer, Department of Surgery, Ann Arbor, MI,[22] and were cultured in DMEM containing 10% FBS and penicillin/streptomycin in humid environment with 5% CO_2 at 37 °C.

2.1. Immunohistochemistry

Immunohistochemical staining (IHC) was performed on sections of formalin-fixed and paraffin-embedded tissue from 18 consecutive cases of EA for which archival tissue was available for evaluation, and on esophageal adenocarcinoma cell lines Bic-1 and Seg-1. Sections were deparaffinized in xylenes, rehydrated through decreasing concentrations of ethanol ending in PBS. For TRAIL-R2 (DR5), antigen retrieval was performed by steam heat treatment of the slides for 8 min in 10 mM citrate buffer, pH 6.0. For TRAIL-R1 (DR4), no antigen retrieval was performed. Sections were then incubated with 1:50 dilution of goat anti-DR4 antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), and 1:50 dilution of rabbit anti-DR5 antibody (IMG-120, Imgenex Corporation, San Diego, CA), in Dako diluent buffer (DakoCytomation, Carpinteria, CA). The bound DR4 antibody was detected using Dako's LSAB plus horseradish peroxidase kit and the bound DR5 antibody was detected using Dako's Envision plus rabbit peroxidase kit, with diaminobenzidine as chromogen. Sections were counterstained with hematoxylin, mounted, and coverslipped. Sections of formalin-fixed and paraffin-embedded Jurkat cells were used as positive controls. Negative controls were sections stained as above, with the exception that they were incubated with Dako's antibody diluent without primary antibody. The percentage of positive cells in tissue sections was determined on a semi-quantitative scale as 0 (completely negative), 1–10%, 11–25%, 26–50%, 51–75%, and >75%. The intensity of immunostaining was recorded as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). Immunostaining results were evaluated by a single pathologist with expertise in immunohistochemistry (M.Y.).

2.2. Western blots

Whole cellular protein was extracted from Bic-1 and Seg-1 human esophageal adenocarcinoma cells and two different samples of human normal peripheral blood lymphocytes (NPL), by incubation in lysis buffer (Cell Signaling Technology, Beverly, MA) for 30 min on ice and then centrifuged to remove cellular debris. The protein in the resulting supernatant was quantified by the BCA method (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions, diluted 2:1 in protein SDS loading buffer (Cell Signaling Technology), and boiled for 5 min. A total of 30 μg of protein was loaded onto 12% Tris–HCl SDS polyacrylamide electrophoresis Ready Gels (Bio-Rad, Hercules, CA), transferred to a nitrocellulose transfer membrane (Bio-Rad), and incubated with mouse anti-TRAIL-R1 antibody (Cat # 40934, Active Motif, Carlsbad, CA) diluted 1:1000, rabbit anti-TRAIL-R2 antibody (Imgenex, Cat # 120) diluted 1:750, or anti-beta actin antibody (Sigma–Aldrich, St. Louis, MO) diluted 1:5000. The bound antibodies were detected by using SuperSignal® West Dura Extended Duration Substrate (Pierce Chemical Co., Rockford, IL).

2.3. Flow cytometry for surface receptor expression

Surface TRAIL-R1 and TRAIL-R2 expression was determined by flow cytometry using fluorescent-labelled anti-TRAIL-R1 and TRAIL-R2 antibodies. Briefly, non-specific binding sites were blocked by preincubation with human non-specific IgG antibody (Sigma Chemicals Co. St. Louis, MO) for 15 min, then incubated for 45 min on ice with 10 μg/ml of anti-TRAIL-R1-PE (IgG_1) and the isotype control IgG_1-PE or the anti-TRAIL-R2-PE (IgG_{2b}) with the isotype control IgG_{2b}-PE (R & D Systems Inc., Minneapolis, MN). Data were collected and analysed on a Becton Dickinson FACSCalibur flow cytometer using CellQuestPro™ software (BD Biosciences, San Jose, CA).

2.4. MTS cell viability assay and apoptosis determination by Annexin V assay

The agonistic monoclonal antibodies against TRAIL-R1 (HGS-ETR1) and TRAIL-R2 (HGS-ETR2) are fully human antibodies of IgG_1 isotype[21–24] and were provided by Human Genome Sciences (Rockville, MD). Antibodies to TRAIL-R1 were obtained from Active Motif, to TRAIL-R2 from Imgenex, and to b-actin from Sigma–Aldrich. The anti-TRAIL-R1-PE (IgG_1) and the isotype control IgG_1-PE as well as the anti-TRAIL-R2-PE (IgG_{2b}) with the isotype control IgG_{2b}-PE were obtained from R & D Systems Inc (Minneapolis, MN). The human IgG_1 lambda chain antibody was obtained from Sigma–Aldrich.

Cells were cultured in 96-well plates at a concentration of 0.1×10^6 cells/mL. Cell viability was assessed a non-radioactive cell proliferation assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS], using CellTiter96® AQ_{ueous} One Solution Reagent (Promega, Madison, WI), as previously described.[18] Briefly, 160 μL of cell suspension and 40 μL of CellTiter96®

AQ_{ueous} One Solution Reagent were incubated in 96-well plates for 1 h at 37°C, 5% CO_2, and formazan absorbance was measured at 490 nm on a μQuant plate reader equipped with KC4 software (Biotek Instruments, Winooski, VT). Each measurement was made in triplicate, and the mean value was determined.

Apoptosis was determined using Annexin-V-FLUOS and propidium iodide double staining (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions and as we have previously published. Data were collected and analysed on a Becton Dickinson FACSCalibur flow cytometer using CellQuestPro™ software (BD Biosciences, San Jose, CA), as published previously.[25]

3. Results

All adenocarcinomas in this study were from male patients. The age ranged from 40 to 88 years (mean 64, median 65). Four tumours were pT1, 3 were pT2, and 11 were pT3. Eleven of the tumours were associated with regional lymph node metastasis. None of the patients received any pre-surgical chemo or radiation therapy.

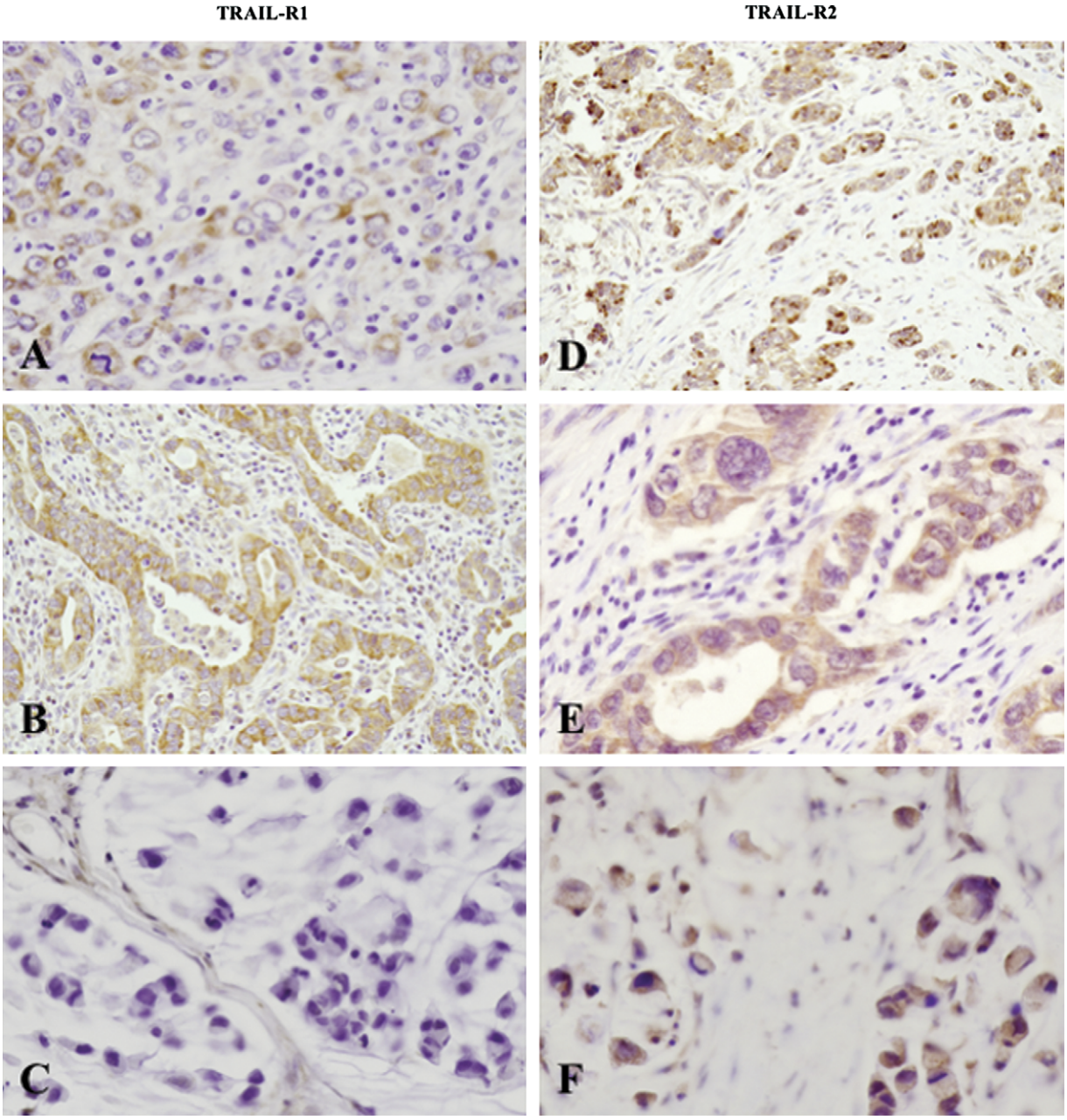

Fig. 1 – TRAIL-R1 and TRAIL-R2 expression in human esophageal adenocarcinoma (EA) tissues: (A) TRAIL-R1 is expressed mainly in the cytoplasm of most tumour cells in a section of poorly differentiated and (B) moderately differentiated EA, but is (C) negative in a mucinous EA. (D and E) TRAIL-R2 is expressed mainly in the cytoplasm of a moderately differentiated EA and (F) mucinous EA.

Sixteen (89%) of the 18 human EA expressed TRAIL-R1 and 17 (94%) expressed TRAIL-R2. TRAIL-R1 expression was moderate to strong, and was present in greater than 50% of the cancer cells in 83% of the cases. TRAIL-R2 expression was moderate to strong in 11 cases and weak in 6, and was present in greater than 50% of the cancer cells in 89% of the cases (Fig. 1). Staining was essentially cytoplasmic in all cases (Fig. 1). Normal squamous epithelium, present in sections of 6 cases, normal esophageal glands, present in sections of 3 cases, and normal muscularis propria were consistently negative for TRAIL-R1 and TRAIL-R2. There was no significant correlation between the percentage of positive cells or intensity of staining with lymph node metastasis or the depth of tumour invasion.

In order to determine whether TRAIL-R1 and TRAIL-R2 are functionally expressed in EA cells, we utilized the human EA cell lines Seg-1 and Bic-1 for this part of our study. Immunohistochemical staining, western blot analysis, and flow cytometry showed expression of TRAIL-R1 and TRAIL-R2 in both cell lines, more TRAIL-R2 than TRAIL-R1 was seen by all these three methods (Fig. 2). Both Seg-1 and Bic-1 expressed 57Kd proteins corresponding to TRAIL-R1 and TRAIL-R2, although TRAIL-R2 appeared to be more abundant in both cell lines (Fig. 2(a)). Immunohistochemical staining showed that TRAIL-R1 is expressed mainly in the cytoplasm of Bic-1 cells (Fig. 2(b1)) and Seg-1 cells (Fig. 2(b3)) with weak to moderate staining of most cells. TRAIL-R2 was expressed mainly in the cytoplasm of Bic-1 (Fig. 2(b2)) and Seg-1 (Fig. 2(b4)) cells with moderate to strong staining, in agreement with the western blot results (Fig. 2(a)). Surface expression of TRAIL-R1 and TRAIL-R2, determined by flow cytometry, was similar to expression of these receptors determined by western blot and IHC, with more TRAIL-R2 present in both cell lines than TRAIL-R1 (Fig. 2(c)).

Bic-1 and Seg-1 cells were then cultured with increasing concentrations of agonistic monoclonal antibodies targeting TRAIL-R1 (HGS-ETR1) and TRAIL-R2 (HGS-ETR2) for different time periods. Cell viability was determined by the MTS assay, and apoptosis was determined by AnnexinV staining and FACS analysis. Both HGS-ETR1 and HGS-ETR2 antibodies induced apoptosis in both Bic-1 and Seg-1 cells in a dose and time dependent manner (Fig. 3). Taken together, our results show that TRAIL-R1 and TRAIL-R2 are expressed in both human EA cell lines and in human EA tissues.

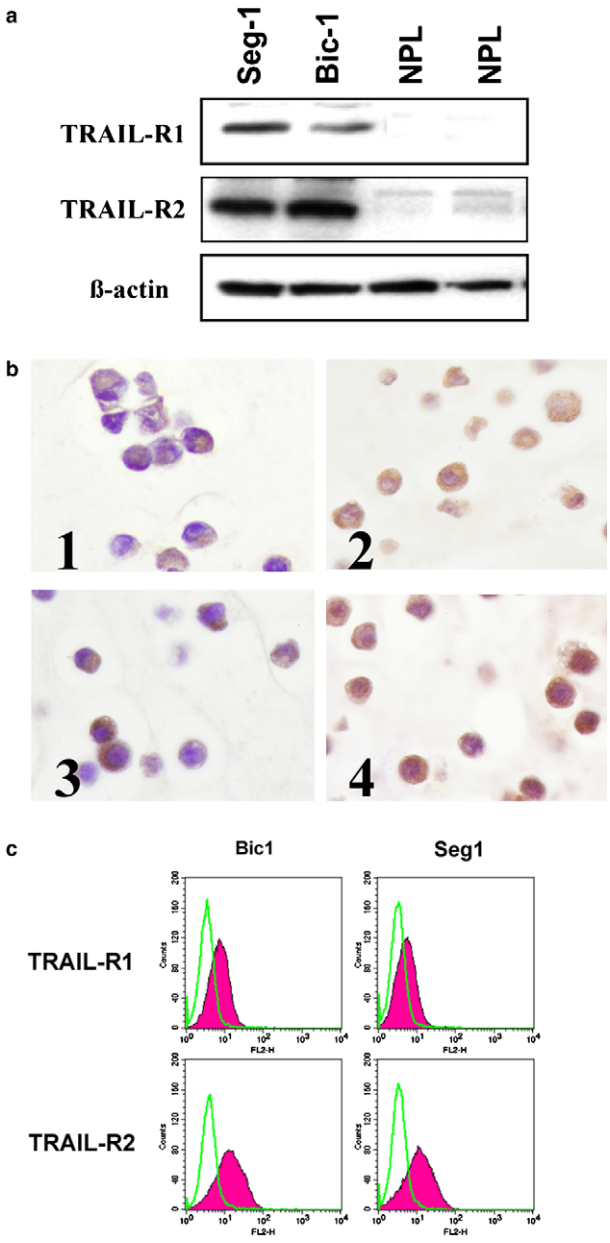

Fig. 2 – TRAIL-R1 and TRAIL-R2 expression in Bic-1 and Seg-1 human esophageal adenocarcinoma cells. Western blots show that both Seg-1 and Bic-1 express 57Kd proteins corresponding to TRAIL-R1 and TRAIL-R2: (a) TRAIL-R2 appears to be more abundant in both cell lines. Immunohistochemical (IHC) staining show TRAIL-R1 is expressed mainly in the (b1) cytoplasm of Bic-1 and (b3) Seg-1 cells with weak to moderate staining of most cells. TRAIL-R2 is expressed mainly in the (b2) cytoplasm of Bic-1 and (b4) Seg-1 cells with moderate to strong staining intensity, in agreement with the western blot results. Surface TRAIL-R1 and TRAIL-R2 expression was determined by flow cytometry using fluorescent-labelled anti-TRAIL-R1 and TRAIL-R2 antibodies. (c) Surface expression of TRAIL-R1 and TRAIL-R2.

4. Discussion

Most human cancer tissues express high levels of TRAIL-R1 and TRAIL-R2 in the majority of cancer cells. Expression of these receptors has been reported previously in a variety of human cell lines and tissues including breast cancer, leukemia, colorectal cancer, pancreatic cancer, and lung cancer.[8,26–31]

Several investigators have shown that antibodies to human TRAIL-R1 and TRAIL-R2 induce apoptosis in a variety of TRAIL-R1 and TRAIL-R2 expressing human cell lines and mouse bearing xenografts.[9,20,32] In one recent study, a monoclonal anti-mouse TRAIL-R2 antibody was found to have potent antitumour activity with no signs of systemic toxicity when tested in vivo in mice[33]. Pukac and colleagues showed that HGS-ETR1, one of the agonistic antibodies we used in this study, reduces the viability of a variety of cancer cell types in vitro by activating both the intrinsic and extrinsic apopto-

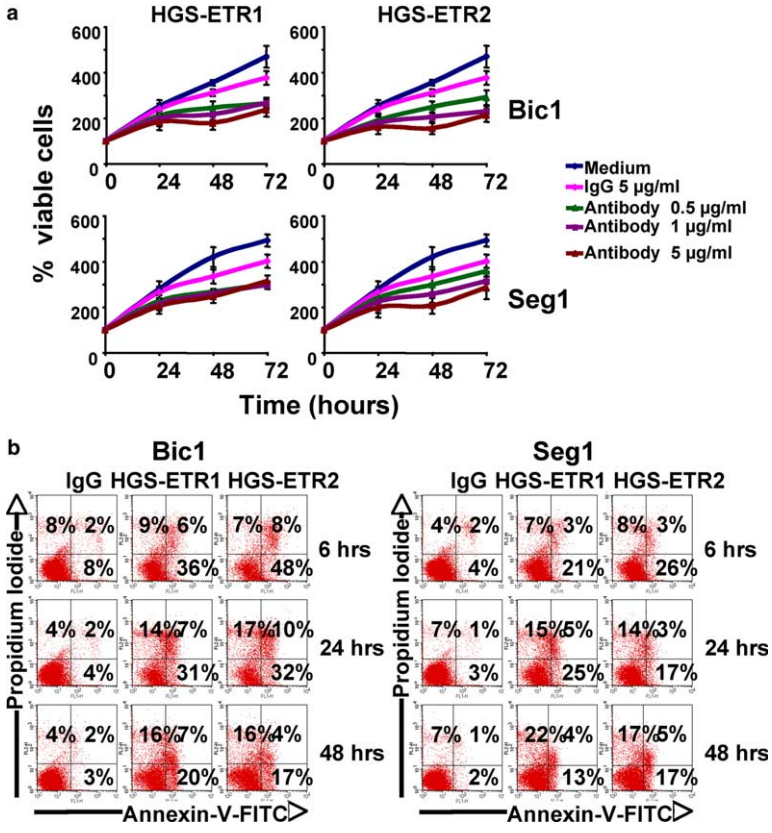

Fig. 3 – The agonistic TRAIL-R1 (HGS-ETR1) and TRAIL-R2 (HGS-ETR2) antibodies reduced cell viability in a time and dose-dependent manner in both Bic-1 cells and Seg-1 cells (a). Values expressed are means ± SD from 3 experiments. Results are expressed as the percentage of viable cells compared to 100% at time 0. Annexin-V-FLUOS and Propidium Iodide double staining, after incubating the cells with 1 μg/ml of normal human IgG, HGS-ETR1 or HGS-ETR2, show that the agonistic antibodies HGS-ETR1 and HGS-ETR2 induced apoptosis in both cell lines (b). In each plot, the percentage of cells in early apoptosis, seen in the right lower quadrant of each square, is higher for the cells treated with the anti-trail receptor antibody than for cells treated with the control non-reactive human IgG.

sis pathways.[34] HGS-ETR1 also induced rapid tumour regression or repression of tumour growth in colon, lung, and renal cancer xenografts following in vivo administration.[34] Furthermore, adding HGS-ETR1 to commonly used chemotherapeutic agents showed enhanced cytotoxicity both in vitro and in vivo.[34] Human agonistic antibodies HGS-ETR1 and HGS-ETR2 are currently in phase I/II clinical trials.

Although in this study we found no expression of TRAIL-R1 and TRAIL-R2 in normal esophageal squamous epithelial cells, Kim and colleagues were able to induce apoptosis in cultured primary normal human esophageal squamous cells using recombinant human TRAIL protein.[35] One possible explanation to this finding is that low levels of TRAIL-R1 and TRAIL-R2 could be expressed in normal keratinocytes below the sensitivity of our detection method but sufficient enough to induce apoptosis in response to activation by TRAIL.

Other possible explanations include that cells may behave differently in vitro than in vivo. It is also possible that these cells no longer resemble the original primary cells.[36] Interestingly, primary cultures of normal human foreskin keratinocytes did not respond to TRAIL.[35]

In the present study we found TRAIL-R1 and TRAIL-R2 to be expressed in the majority of human EA tissues. We also found that agonist anti-TRAIL-R1 and TRAIL-R2 antibodies induce apoptosis in human EA cell lines expressing these receptors in a time and dose dependent manner. Our findings suggest that human EAs, which are associated with very poor prognosis and to which there is no accepted effective non-surgical treatment, may be good candidates for treatment with agonist antibodies targeted to TRAIL-R1 and TRAIL-R2.

Conflict of interest statement

None declared.

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