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Receptor activator of nuclear factor kappaB (RANK) is expressed as a late event during malignant progression in Barrett's metaplasia

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Abstract

Receptor activator of NF-kappaB (RANK) activation induces several survival signals including nuclear factor kappaB (NF-kappaB) and activation and upregulation of Bcl-xL. The aim of this work was to determine whether RANK is expressed in oesophageal adenocarcinoma (EA) and its precursor, Barrett's metaplasia (BM). Sections of formalin-fixed and paraffin-embedded tissue from 23 oesophagectomy specimens showing EA and variable degrees of dysplasia in BM were immunohistochemically stained for RANK. All 23 carcinomas (100%) showed strong RANK immunoreactivity in most cancer cells. In 8 cases with high-grade dysplasia (HGD), RANK immunoreactivity was weaker and detected in a smaller percentage of cells. RANK was not detected in any area of BM that was negative for dysplasia (ND) (17 cases), or showed low-grade dysplasia (LGD) (9 cases). These results indicate that RANK is expressed as a late event during malignant progression in BM. Studies are needed to determine whether interfering with RANK signalling affects the survival of EA cells.

Keywords: Barrett's oesophagus; Metaplasia; Cancer; Immunohistochemistry; RANK

1. Introduction

Receptor activator of nuclear factor (NF)-kappaB (RANK) is a member of the tumour necrosis factor (TNF)-receptor superfamily that shares the highest sequence homology with the extracellular domain of CD40 [1]. RANK mRNA is ubiquitously expressed in human tissues, but RANK protein expression has been detected only in dendritic cells, activated T lymphocytes, and osteoclast haematopoietic precursor cells, suggesting that expression of the protein is transcriptionally regulated [1,2]. Like other TNF receptor family members, RANK activates several signalling pathways by interacting with various TNF-receptor-associated factors (TRAFs) [3–5]. The signalling pathways activated by RANK include NF-kappaB, c-jun amino-terminal kinase and c-Src [6]. The physiological function of RANK has been demonstrated in knockout experiments in which RANK$^{-/-}$ mice showed profound osteopetrosis, lack of lymph node formation, and B-cell deficiency in the spleen [7]. Other studies have indicated that RANK can provide survival signals for dendritic cells, perhaps due to the upregulation of the anti-apoptotic protein, Bcl-xL [8]. Furthermore, RANK activation has been reported to induce cytokine secretion (interleukin-1, interleukin-6 and interleukin-12) [9] and to be involved in mammary gland development [10] and in angiogenesis [11]. NF-kappaB has been shown to play an important role in the proliferation, survival and apoptosis of epithelial cells and several human malignancies [12–17]. The expression of RANK and its natural ligand RANKL (also called TRANCE) in human tumour cells remains poorly studied, although a recent report demonstrated functional expression of this ligand/receptor pair in Hodgkin's lymphoma [18]. We undertook this study to determine whether RANK is expressed in oesophageal adenocarcinoma (EA), and its precursor lesion Barrett's metaplasia (BM), with or without dysplasia.

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2. Patients and methods

This study was approved by the Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals.

Sections of formalin-fixed and paraffin-embedded tissue from 23 oesophagectomy specimens showing EA and a variable degree of dysplasia in BM were immunohistochemically stained for RANK using a standard immunoperoxidase method. In the same sections from the carcinoma cases, BM that was negative for dysplasia (ND) was present in 17 of the cases, low-grade dysplasia (LGD) was observed in 9 cases, and high-grade dysplasia (HGD) in 8 of the cases.

Of the 23 patients, 22 were males and 1 was a female, with ages ranging from 40 to 88 years (mean 63 years, median 64 years). Four (17%) of the tumours were T1, three (13%) were T2, 15 (65%) were T3, and one (4%) was T4. Five (22%) tumours were node-negative, 16 (70%) were node-positive, and in two (9%) no lymph nodes were found in the resected specimen. One tumour (4%) was well differentiated, 12 (52%) were moderately differentiated, and 10 (43%) were poorly differentiated.

Immunostaining was performed at room temperature utilising a Dako Autostainer (Dako Corporation, Carpinteria, CA, USA), and the wash buffers supplied by DAKO. Briefly, following deparaffinisation and rehydration, sections were subjected to heat-induced antigen retrieval in 10-mM citrate buffer, then incubated with 1:100 dilution of the polyclonal rabbit anti-RANK antibody (H300, sc-9072, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 20 min. The bound antibody was detected using an Envision plus rabbit peroxidase kit (Dako) with diaminobenzidine as the chromogen. Finally, sections were counterstained in haematoxylin, dehydrated, mounted and coverslipped. Positive controls were sections of formalin-fixed and paraffin-embedded cell lines, HD-MYZ and HDLM-2, which we have shown to express RANK [18], and negative controls were sections immunostained as above, but utilising a non-relevant antibody of the same species as the primary antibody. The percent of positive cancer cells in each case was scored separately for the carcinomas, BM ND, LGD and HGD, on a semi-quantitative scale as follows: (1) 0%; (2) 1–10%; (3) 11–25%; (4) 26–50%; (5) 51–75% and (6) $>75\%$.

Grading of dysplasia was performed according to previously published criteria [19]. Statistical analysis was performed using the Fisher's exact test utilising InStat statistical software, version 3.0a (GraphPad Inc., San Diego, CA, USA).

3. Results

None of the areas of that were BM ND or LGD showed positive RANK staining. In these cases, there was a non-specific staining uptake by the mucinous material in the goblet cells, a common artifact in immunohistochemistry (Fig. 1a).

Interestingly, many stromal cells and lymphocytes also expressed RANK. In the positive cases of HGD, RANK immunoreactivity was weak and cytoplasmic (Fig. 1b). By contrast, in cases of EA strong cytoplasmic RANK, immunoreactivity with membranous accentuation was observed in all cases (Fig. 1c). All 23 EA (100%) showed strong RANK immunoreactivity. This was detected in $>75\%$ of the cancer cells in 14 (61%) cases, 51–75% of the cells in 7 (30%) cases, 26–50% of the cells in 1 (4%) case, and in 11–25% of the cancer cells in 1 (4%) of the cancer cases. In areas of HGD from the 8 cases, RANK was detected in $>75\%$ of the dysplastic cells in 1 (13%) case, in 51–75% of the cells in 2 (25%) cases, in 11–25% of the cells in one (13%) case, in $\leqslant 10\%$ of the cells in 3 (38%) cases, and was completely negative in 1 (13%) case. RANK was not detected in any of the areas of the 17 cases of BM ND (0%), or the 9 cases LGD (0%).

There was no significant difference in the percentage of RANK-positive cancer cells with regard to the nodal status, depth of tumour invasion (T1 and T2 versus T3 and T4) or grade (well and moderately differentiated versus poorly differentiated).

4. Discussion

Since the 1970s, the incidence of EA in the United States (US) has been steadily rising in both males and females and whites and hispanics [20]. Although survival has been improving, it remains poor, with only 25% of patients being alive 3 years after resection [20]. This is despite the advances in cancer diagnosis and treatment that has been made in recent decades. New markers of malignant transformation that can help with early detection, as well as new targets for therapy, are needed in order to improve the survival rates of patients with this cancer.

In the present study, we have demonstrated that RANK is overexpressed as a late event during the malignant progression in BM, being undetectable in BM without dysplasia or in LGD. However, it is not possible from this limited study to determine whether RANK expression can distinguish between the different grades of dysplasia, or whether it can distinguish dysplastic from reactive changes associated with inflammation. This will need a larger study with adequate follow-up, which is currently in progress in our laboratory.

RANK activation has been shown to transduce several intracellular survival pathways. Although no link has been established between RANK expression and malignant transformation, activation of NF-kappaB, a target of RANK signalling, has been implicated in

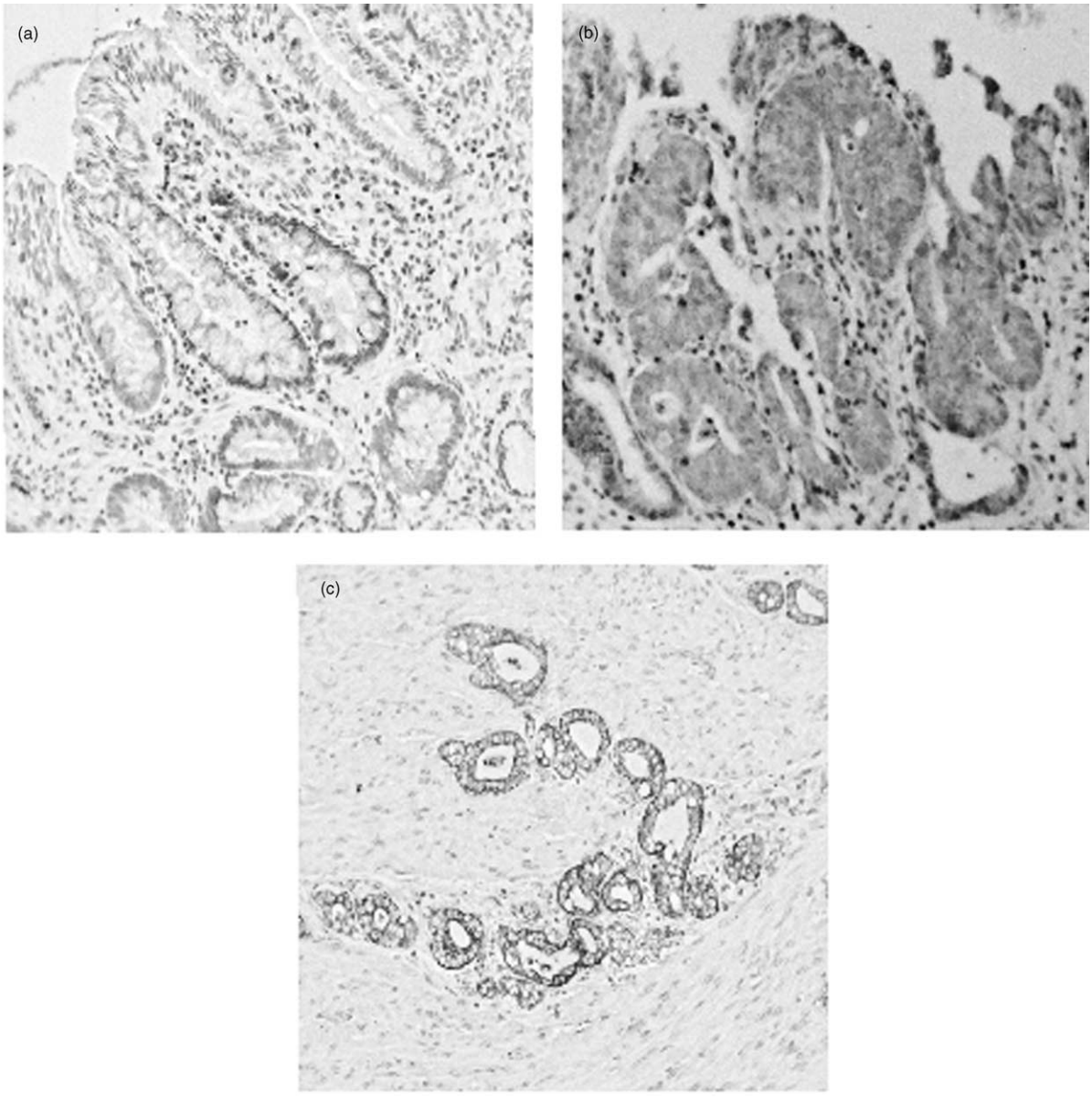

Fig. 1. Immunoperoxidase staining for RANK in Barrett's metaplasia (BM) and oesophageal adenocarcinoma (EA). A BM without dysplasia is negative for RANK; the mucin present in the goblet cells shows a faint non-specific staining (A). Weak cytoplasmic staining can be detected in a BM with high-grade dysplasia (HGD) (B), but strong cytoplasmic RANK immunoreactivity with membranous accentuation is usually seen in EA (C). Many lymphocytes in the stroma are RANK-positive (A and B).

malignant transformation [21–23]. Since not all cases with dysplasia in BM progress to EA [24,25], RANK overexpression may be an important event at the final step of the malignant transition from HGD to carcinoma. Prospective studies are needed to determine whether cases of HGD and RANK overexpression are more likely to progress to invasive EA. NF-kappaB plays an important role in carcinogenesis and in tumour growth, metastasis and survival [13,15,16,26]. Inhibition or suppression of NF-kappaB activity results in apoptosis, increased sensitivity to apoptosis-inducing agents, and inhibition of malignant transformation in many tumour types [14,17,19,27–31]. Additional studies are needed to determine the biological significance of RANK overexpression in EA.

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