The Veterinary Journal 198 (2013) 131–140

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Immunohistochemical expression of Bax and Bak in canine non-neoplastic tissues

Martina Croci, Martina Dettwiler, Lloyd Vaughan, Franco Guscetti *

Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 268, 8057 Zurich, Switzerland

ARTICLE INFO

Article history:
Accepted 21 July 2013

Keywords:
Apoptosis
Bak
Bax
Dog
Immunohistochemistry
Western blot

ABSTRACT

Apoptosis is critical for embryonic development, maintenance of tissue homeostasis and protection against malignant transformation. The Bcl-2 family of proteins plays a key role in intrinsic apoptosis by controlling the integrity of the outer mitochondrial membrane, and the multidomain pro-apoptotic Bcl-2 family members Bax and Bak are essential components of this pathway. The aim of this study was to provide data on the expression of these proteins in normal canine tissues. Two antibodies against Bax recognising different conformations of the protein and one antibody against Bak were validated by immunohistochemistry and immunoblotting using canine recombinant proteins and keratinocytes treated with ultraviolet light. The antibodies were used immunohistochemically to label a wide panel of histologically normal tissues assembled on tissue microarrays. In addition, a subset of the tissues was evaluated by Western blot analysis.

Immunohistochemical and Western blot analyses revealed that both Bax and Bak are widely expressed in non-neoplastic tissues from adult dogs. Immunohistochemistry showed almost exclusively cytoplasmic labelling and prominent labelling of epithelial cells. In lymph nodes, immunohistochemical labelling was diffuse for both proteins and showed enhanced intensities in the mantle zones for Bax and the germinal centres for Bak. Strong reactivity for the active conformation of Bax was detected only in enterocytes and Leydig cells and in scattered lymphocytes. These data indicate widespread expression of Bax and Bak in normal canine tissues. Knowledge of the expression of Bax and Bak in normal tissues is a prerequisite in assessing the role of these proteins in canine neoplastic disease.

© 2013 Elsevier Ltd. All rights reserved.

Introduction

Apoptosis is a genetically controlled cell death program critical for embryonic development, maintenance of tissue homeostasis and protection against malignant transformation (Cory et al., 2003; Elmore, 2007). Two main apoptotic pathways are known, namely, the extrinsic or death-receptor pathway, which is triggered by extracellular ligands, and the intrinsic or mitochondrial pathway. Intrinsic apoptosis is induced by many different intracellular signals and is regulated by the Bcl-2 protein family, which controls the integrity of the outer mitochondrial membrane (OMM) through interactions between members of its three major subgroups. One group comprises the effector multidomain pro-apoptotic members Bax (Bc-l2-associated X protein) and Bak (Bc-l2-homologous antagonist/killer) that are gateway proteins essential to intrinsic apoptosis (Wei et al., 2001).

Following an adequate pro-apoptotic stimulus, Bax and Bak are activated, change their conformation and Bax then translocates from the cytoplasm to the OMM, where Bak already resides. There they form homo-oligomeric pores which allow for the release into the cytosol of cytochrome c and other proteins that induce the demise of the cell (Letai, 2008). The members of the anti-apoptotic subgroups (with Bcl-2 as the prototype) can bind Bax and Bak hindering their activation. Proteins of the third subgroup, the BH3-only proteins, inhibit the anti-apoptotic proteins and, under some circumstances, can directly activate Bax or Bak (Cory et al., 2003).

Studies with knock-out animals suggest an extensive functional overlap between Bax and Bak but also that their combined functions are essential for normal development (Knudson et al., 1995; Lindsten et al., 2000). Expression studies in humans based on mRNA or protein assays, including immunohistochemistry, point to a widespread tissue distribution of these two proteins (Oltvai et al., 1993; Krajewski et al., 1994, 1996; Kiefer et al., 1995). This notion is supported by immunohistochemical data from a publicly accessible repository (The Human Protein Atlas[1]). Accordingly, Bax

* Corresponding author. Tel.: +41 44 635 85 95.
E-mail address: franco.guscetti@vetpath.uzh.ch (F. Guscetti).

[1] The Human Protein Atlas, http://www.proteinatlas.org (version 11, accessed August 2011).

1090-0233/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tvjl.2013.07.029

is most strongly expressed in the epithelial cells of the gastrointestinal tract and trachea, urothelium, renal tubules, breast, and prostate gland, in the testis and in endocrine glands (thyroid, parathyroid and adrenal). Similarly, Bak is most strongly expressed in epithelial cells of the epidermis, gastrointestinal tract and trachea, urothelium, as well as in the lymphatic system and adrenal gland (Oltvai et al., 1993; Krajewski et al., 1994, 1996; Kiefer et al., 1995; The Human Protein Atlas[1]).

Deregulation of apoptosis is a recognised mechanism contributing to tumourigenesis and resistance to therapy. Three specific blocks of apoptosis have been postulated at the level of the Bcl-2 family of proteins, including: (1) overexpression of anti-apoptotic members; (2) functional impairment or expression loss of key BH3-only proteins; or (3) functional impairment or expression loss of Bax and Bak (Letai, 2008). Thus, knowledge about the expression of these proteins in non-neoplastic tissues is necessary to characterize their role in tumourigenesis.

To our knowledge, there are no immunohistochemical data available yet on the expression of Bax and Bak in normal canine tissues. The present tissue microarray (TMA)-based study using thoroughly validated antibodies against total Bax, activated Bax and total Bak, contributes to closing this gap. Catalogues of the immunohistochemical expression of these proteins, partially validated through Western blot analyses, are provided for future reference, especially to support oncology studies.

Materials and methods

Recombinant proteins

cDNAs coding for for canine Bak (GenBank accession number AAY19401) and Bax (sequence identical to GenBank BAC56139) cloned into a pGEX4t2 vector (Invitrogen) were available from a concomitant study (S. de Brot et al., unpublished data). They were expressed as fusion proteins with N-terminal glutathione S-transferase (GST) in BL21 Star *Escherichia coli* (Invitrogen) as described previously (Keller et al., 2007). For Western blot analysis, lysates from liquid cultures of bacteria expressing GST-Bak or GST-Bax were purified using glutathione Sepharose beads as described previously (Wimmershoff et al., 2010). For immunohistochemistry, bacteria fixed for 24 h in 4% neutral buffered formaldehyde were embedded in paraffin wax (Wimmershoff et al., 2010). To create a representative array, cylinders of 0.6 mm diameter (cores) of each paraffin block containing bacteria were transferred to a recipient block using a manual tissue arrayer (Beecher Instruments).

Cultured keratinocytes

Canine normal keratinocytes (Bskin cell line) were grown in Dulbecco's Modified Eagles medium (DMEM), irradiated with 100 mJ UVC light using a cross linker, harvested and processed for Western blot analysis and immunohistochemistry as described by Dettwiler et al. (2013).

Canine tissues

For immunohistochemical analysis, TMAs containing normal tissues originating from at least three different adult dogs were available (Wimmershoff et al., 2010). Two additional TMAs were assembled with various tissues and lymph nodes, respectively, all freshly sampled from seven additional dogs that had been euthanased in the context of an unrelated experiment and were free of neoplastic disease.

All tissues were fixed in 4% neutral buffered formaldehyde for 24 h, routinely embedded into paraffin wax, and were free of histopathological lesions (i.e. normal). Whole sections of skin and lymph nodes were used to confirm TMA findings. For Western blot analysis, selected tissues of these dogs were snap frozen in liquid nitrogen and stored at −80 °C.

Antibodies

Commercially available antibodies against human Bax and Bak were selected based on homology of the immunogen with the canine sequences and/or reactivity with recombinant canine proteins. They included: (1) a rabbit polyclonal anti-Bax antibody clone A20 (DB005, Delta Biolabs; immunogen: amino acids (aa) 11–30) denominated henceforth BAX-AbA20; (2) a mouse monoclonal anti-BAX antibody clone 2D2 (MS-711-P0, NeoMarkers; immunogen: aa 3–16) called henceforth BAX-Ab2D2; and (3) a polyclonal anti-Bak antibody 06-536 (Upstate; immunogen: aa 23–38) denominated henceforth Bak-AbNT. In addition, an anti-β-actin antibody ab8227 (Abcam) was used to normalize Western blot results.

Western blots

The procedure was performed as described by Dettwiler et al. (2013) with slight modifications. Equal amounts (100 µg) of protein for each sample were determined using NanoDrop (Thermo Fisher Scientific) and diluted in a sodium dodecyl sulfate (SDS) loading buffer, run in 15% SDS gels and blotted onto PVDF membranes. Primary antibodies Bak-AbNT (0.5 µg/mL) or BAX-AbA20 (0.5 µg/mL) or BAX-Ab2D2 (0.4 µg/mL) or ab8227 (0.3 µg/mL) were applied for either 1 h at room temperature or overnight at 4 °C. Polyclonal and monoclonal antibodies were diluted in Tris-buffered saline and 0.1% Tween 20 (TBST) containing 1% skimmed milk or 1% BSA, respectively. Secondary antibodies, either goat anti-mouse-horseradish peroxidase (HRP)-labelled (Geno Technologies) or goat anti-rabbit-HRP-labelled (Jackson ImmunoResearch), were applied at a 1:7500 dilution. Negative controls included omitting the primary antibody and antibody preincubation as described below in the antibody preincubation section.

Immunohistochemistry

Sections (2 µm) were deparaffinized, rinsed in deionised water and subjected to antigen retrieval consisting of heating in a steamer (Pascal S2800, Dako) in EDTA buffer, pH 9.0, for 20 min at 98 °C (Bak-AbNT and BAX-AbA20), and for 2 min at 125 °C (BAX-Ab2D2). Primary antibodies diluted in Antibody Diluent (S2002, Dako) were incubated in a moist chamber 1 h at room temperature for BAX-Ab2D2 (2 µg/mL) and Bak-AbNT (0.5 µg/mL) or overnight at 4 °C for BAX-AbA20 (2 µg/mL). Peroxidase-blocking solution (S2023, Dako) was applied for 10 min. The signal was visualized using the DAKO Detection kit (Detection system, Dako) according to the manufacturer's instructions. Negative controls included omitting the primary antibody and antibody preincubation as described in the next section. The immunohistochemical reactions were scored based on labelling intensities as follows: 0 = signal absent; 0.5 = very weak; 1 = weak; 2 = moderate; and 3 = strong signal.

Antibody preincubation

The polyclonal antibodies Bak-AbNT and BAX-AbA20 were mixed by inversion overnight at 4 °C with purified GST-Bak or GST-Bax at a 100:1 antigen–antibody ratio, as previously described (Wimmershoff et al., 2010) and used as pre-incubated negative control for Western blots and immunohistochemistry.

Results

The antibodies against human Bax and Bak (polyclonal BAX-AbA20 and Bak-AbNT and monoclonal BAX-Ab2D2) also recognised canine Bax and Bak expressed as glutathione-S-transferase (GST) fusion proteins in bacteria. In Western blots (Fig. 1A), all antibodies labelled bands corresponding to the full-length products (Bax, 55 kDa; Bak, 57 kDa, although the latter product was inconsistently present) and degradation products (as deduced from reactivity with an anti-GST antibody, not shown). Furthermore, immunohistochemistry with formalin-fixed, paraffin-embedded bacteria showed that all antibodies specifically reacted with formalin-resistant canine epitopes (Fig. 1B).

Upon staining keratinocytes, BAX-AbA20 elicited a weak cytoplasmic punctate to diffuse signal that was enhanced after UV treatment (Fig. 1C). BAX-Ab2D2 elicited no signal in untreated, non-apoptotic cells and a strong, granular cytoplasmic signal in treated apoptotic cells (Fig. 1C). Western blot analysis with BAX-Ab2D2 revealed a specific band (21 kDa) that was more pronounced after irradiation (Fig. 1D). Bak-AbNT showed a cytoplasmic punctate immunohistochemical signal that was enhanced after UV-treatment (Fig. 1C). Western blot analysis revealed a specific band (24 kDa) that was enhanced in treated cells (Fig. 1D). Data from a detailed time course experiment documenting enhancement of the signals after UV-treatment are reported in Appendix A (Supplementary material).

The antibodies were specific for canine Bax and Bak and labelled these proteins at physiological levels with labelling intensities apparently proportional to the intracellular protein amounts detected. More importantly, BAX-Ab2D2 appeared to label

Download English Version:

https://daneshyari.com/en/article/5798467

Download Persian Version:

https://daneshyari.com/article/5798467

Daneshyari.com