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TNF-α induced secretion of HMGB1 from non-immune canine mammary epithelial cells (MTH53A)

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ABSTRACT

Background: Mammary neoplasias are one of the most frequent and spontaneously occurring malignancies in dogs and humans. Due to the similar anatomy of the mammary gland in both species, the dog has become an important animal model for this cancer entity. In human breast carcinomas, the overexpression of a protein named high-mobility group box 1 (HMGB1) was reported. Cells of the immune system were described to release HMGB1 actively exerting cytokine function. Thereby it is involved in the immune system activation, tissue repair, and cell migration. Passive release of HMGB1 by necrotic cells at sites of tissue damage or in necrotic hypoxic regions of tumors induces cellular responses e.g. release of proinflammatory cytokines leading to elevated inflammatory response and neo-vascularization of necrotic tumor areas.
Herein we investigated if a time-dependent stimulation with the separately applied proinflammatory cytokines TNF-α and IFN-γ can cause secretion of HMGB1 in a non-immune related HMGB1-non-secreting epithelial canine mammary cell line (MTH53A) derived from non-neoplastic tissue.
Methods: The canine cell line was transfected with recombinant HMGB1 bicistronic expression vectors and stimulated after transfection with the respective cytokine independently for 6, 24 and 48 h. HMGB1 protein detection was performed by Western blot analysis and quantified a by enzyme-linked immunosorbent assay. Live cell laser scanning multiphoton microscopy of MTH53A cells expressing a HMGB1–GFP fusion protein was performed in order to examine, if secretion of HMGB1 under cytokine stimulating conditions is also visible by fluorescence imaging.
Results: The observed HMGB1 release kinetics showed a clearly time-dependent manner with a peak release 24 h after TNF-α stimulation, while stimulation with IFN-γ had only small effects on the HMGB1 release. Multiphoton HMGB1 live cell microscopy showed diffuse cell membrane structure changes 29 h after cytokine-stimulation but no clear secretion of HMGB1–GFP after TNF-α stimulation was visible.
Conclusion: Our results demonstrate that non-immune HMGB1-non-secreting cells of epithelial origin derived from mammary non-neoplastic tissue can be induced to release HMGB1 by single cytokine application. This indicates that tumor and surrounding tissue can be stimulated by tumor present inflammatory and necrotic cytokines to release HMGB1 acting as neo-vascularizing factor thus promoting tumor growth.

1. Introduction

Human and canine mammary tumors are one of the most frequent malignancies in females. In both species they present similar patterns of tumor progression and metastasis [1]. Thereby, the dog has become an important animal model for cancer due to the fact that tumors in dogs are naturally occurring in contrast to the commonly used rodent animal model systems with induced tumors [2]. Accordingly, several distinct naturally occurring canine cancers

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were reported to show similar biological behavior when compared to their human counterpart e.g. mammary carcinomas, osteosarcomas or lung carcinomas [3,4]. In case of mammary gland tumors a clear age and hormone dependency has been shown in dogs as well as in humans [5,6]. The anatomy of the mammary gland in both species is very similar, making the dog an excellent model to reveal mechanisms of the respective tumor biology and to evaluate therapeutic approaches providing benefit to both species.

One protein strongly associated with breast cancer in dogs and humans is the high-mobility group box protein 1 (HMGB1; syn. amphoterin or HMG-1). This protein has an amino acid sequence identity of 100% amongst the two species [7]. Therefore it provides an interesting aspect in terms of comparative oncology.

HMGB1 was reported to be higher expressed in primary human breast carcinomas in comparison to normal breast tissue [8,9]. Analysis of HMGB1 transcripts by Northern blot hybridization identified a strong intertumoral variation of *HMGB1* expression among breast cancer samples [9].

HMGB1 was initially described as an ubiquitous expressed nonhistone nuclear protein belonging to the HMG-protein-superfamily [10,11]. Later on, HMGB1 was discovered to exert two main fields of activity depending on the cellular localization. Intracellular HMGB1 plays a role as DNA-binding protein to sustain nucleosome structure [12], acts as architectural transcription factor regulating gene expression by bending promoter DNA regions [13] and modulates activity of steroid hormone receptors [14].

Besides the nuclear function, HMGB1 can be passively or actively released into the extracellular space acting there as a proinflammatory cytokine. Passive liberation of HMGB1 is caused by necrotic or damaged cells [15,16]. In contrast, cells that undergo apoptosis do not secrete HMGB1 as the protein stays tightly associated to the chromatin [15]. HMGB1 is actively secreted by activated cells of the immune system such as monocytes, macrophages, pituicytes, dendritic cells and natural killer cells [17–21]. Those cells release HMGB1 in response to inflammatory stimuli causing cellular responses like inflammation, sepsis, development of acute lung injury as well as stimulation of cytokine expression and secretion [17,18,22,23]. In macrophage cell cultures, HMGB1 is found to be released as a "late" appearing inflammatory mediator in a delayed manner 8–20 h after bacterial infection or following stimulation with endotoxin or "early" cytokines (tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interferon-gamma (IFN-γ)) [22,24–26].

In addition to immune-related cells, adenocarcinoma derived tumor cell lines (HCT 116, WiDr, Caco-2) were also reported to secrete HMGB1 spontaneously without exogenous stimuli or in a polarized fashion after cytokine-mix stimulation [27–29]. Liu et al. were able to induce HMGB1 secretion in primary mouse intestinal epithelial cells (IEC) after stimulation with a mixture of different cytokines [29]. Fujii et al. recently demonstrated that also co-treatment with chemical factors (deoxycholic acid and azoxymethane) leads to accelerated secretion of HMGB1 in IEC-6 cells [30].

However, the relationship between HMGB1-release and time-dependently stimulation with the separately applied inflammatory cytokines TNF-α und IFN-γ in a mammary epithelial cell line has not been studied so far.

The effect of extracellular HMGB1 is mediated by interaction with the receptor for advanced glycation end products (RAGE) [31] and/or the Toll-like receptors (TLR)-2, and -4 [32,33]. Signaling via Toll-like receptors enables cells of the innate immune system to respond to various exogenous and endogenous stimuli causing cell activation and transcription of proinflammatory cytokines [34]. In the setting of cancer, the HMGB1/RAGE ligand/receptor complex is supposed to regulate proliferation, migration and metastasis of tumor cells [31,35,36].

Due to the high mitotic rate of the tumor cells and the existence of unsystematic tumor vessel architecture, hypoxia can be observed in large numbers of solid tumors [37] leading to the formation of necrotic areas with passively released HMGB1 [38]. In this scenario HMGB1 is supposed to play a key role in the promotion of the tumor growth by mediating angiogenic effects through increasing the expression of angiogenesis-related cytokines and growth factors [38,39]. Besides necrotic tumor cells, activated tumor-associated macrophages have the function to release HMGB1 actively as response to hypoxia or cytokines (e.g. TNF-α or IL-1β) and also by HMGB1 itself due to autostimulation [17,18,22,23].

The mediation of HMGB1 signaling between non-tumorigenic cells like e.g. epithelial cells and the tumor cells in case of breast cancer is supposed to be arranged by specialized activated tumor stroma [40].

Therefore, we hypothesized in the described context of tumor development and progression if also non-transformed HMGB1-non-secreting epithelial cells transfected with recombinant HMGB1 bicistronic expression vectors can be stimulated by time-dependent separate application of the proinflammatory cytokines TNF-α and IFN-γ to start active HMGB1 secretion.

To prove this, we chose a non-neoplastic HMGB1-non-secreting MTH53A canine mammary cell line of epithelial origin for our experiments with regard to the previously described advantages of the canine animal model resembling human neoplasias.

We were able to detect a clear secretion of recombinant HMGB1 from TNF-α stimulated MTH53A cells by HMGB1–Western blotting and quantified the amount of released HMGB1 by enzymelinked immunosorbent assay (ELISA). To confirm the results gained by Western blotting and HMGB1-ELISA, we performed multiphoton live cell microscopy on transfected HMGB1–GFP expressing MTH53A cells under TNF-α stimulating condition to visualize the secretion of HMGB1.

2. Materials and methods

2.1. pIRES-hrGFPII plasmid construction

Two variants of recombinant pIRES-hrGFP II (Stratagene, La Jolla, CA) vector constructs were generated, carrying the *hrGFP* gene (pIREShrGFP) and the gene of interest.

The bicistronic expression cassette of the different constructed vectors permits the simultaneous but separate expression of the inserted genes of interest and of *hrGFP* showing successful transfection of the cells with the respective vector by green fluorescence.

The first variant contains the canine *HMGB1* coding sequence (GenBank ID: AY135519.1) without the terminal stop codon resulting in an HMGB1 fusion protein with a recombinant short 3×FLAG peptide sequence at its C-terminal part (pIRES-hrGFP II-*rHMGB1* + FLAG) to differentiate via immunohistochemistry between endogenous and expressed recombinant HMGB1 protein (rHMGB1 + FLAG). The second construct contains the complete HMGB1 coding sequence, including the terminal TAA stop codon (pIRES-hrGFP II-*rHMGB1*). This vector variant was constructed to make sure that the 3×FLAG-tail has no effect on the function of the recombinant HMGB1 (rHMGB1) protein.

The following primer pairs were used for polymerase chain reaction (PCR) to amplify the corresponding sequences: EcoRI-B1-CFA-FWD (5′- GGAATTCCACCATGGGCAAAGGAGA-3′; forward primer for rHMGB1 + FLAG and rHMGB1), NotI-B1-CFA-REV (5′-GCGCGGCCGCTTATTCATCATCATC-3′, reverse primer rHMGB1), NotI-B1-CFA-Rev/-TAA (5′-AAGAATGATGATGATGAAGCGGCCGCG C-3′, reverse primer rHMGB1 + FLAG). The amplified PCR products were separated on a 1.5% agarose gel, eluted using QIAquick Gel