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The receptor for advanced glycation end products influences the expression of its S100 protein ligands in melanoma tumors

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ABSTRACT

Recent studies have suggested that the receptor for advanced glycation end products (RAGE) participates in melanoma progression by promoting tumor growth. However, the mechanisms of RAGE activation in melanoma tumors are not clearly understood. To get deeper insights into these mechanisms, we transfected a melanoma cell line, which was established from a human melanoma primary tumor, with RAGE, and studied the effect of RAGE overexpression on cell proliferation and migration *in vitro*. We observed that overexpression of RAGE in these cells not only resulted in significantly increased migration rates compared to control cells, but also in decreased proliferation rates (Meghnani et al., 2014).

In the present study, we compared the growth of xenograft tumors established from RAGE overexpressing WM115 cells, to that of control cells. We observed that when implanted in mice, RAGE overexpressing cells generated tumors faster than control cells. Analysis of protein tumor extracts showed increased levels of the RAGE ligands S100B, S100A2, S100A4, S100A6 and S100A10 in RAGE overexpressing tumors compared to control tumors. We show that the tumor growth was significantly reduced when the mice were treated with anti-RAGE antibodies, suggesting that RAGE, and probably several S100 proteins, were involved in tumor growth. We further demonstrate that the anti-RAGE antibody treatment significantly enhanced the efficacy of the alkylating drug dacarbazine in reducing the growth rate of RAGE overexpressing tumors.

1. Introduction

The receptor for advanced glycation end products (RAGE) is a pattern recognition receptor that has been suggested to be involved in many human diseases including cancer (Alexiou et al., 2010; Schmidt et al., 2000; Sparvero et al., 2009). Several research groups, including ours, have investigated the role of RAGE in melanoma (Abe et al., 2004; Hsieh et al., 2003; Leclerc, 2011; Leclerc et al., 2009b; Saha et al., 2010). Abe et al. (2004) showed that RAGE was expressed at higher levels in the two melanoma cell lines A375 and G361 than in melanocytes. In a different study, Hsieh et al. showed that 20% of analyzed human melanoma tumor specimens stained positive for RAGE, suggesting that not all melanoma tumors express RAGE (Hsieh et al., 2003). Heterogeneity in RAGE expression among melanoma tumors was also suggested by our study where we found up to 50-fold differences in RAGE transcript levels among 40 melanoma tumor samples (Leclerc et al., 2009b).

In an effort to gain a better understanding of the role of RAGE in melanoma, we previously stably transfected WM115 human melanoma cells, which were established from a human primary melanoma tumor, with RAGE, and studied the effect of RAGE overexpression on the cellular proliferation and invasion of these cells (Meghnani et al., 2014). Our study showed that cell migration was significantly enhanced in RAGE overexpressing cells, at the expense of cell proliferation, which was significantly reduced (Meghnani et al., 2014). Comparison of the organization of the actin filaments in the two cell type variants (WM115-RAGE and WM115-MOCK) further suggested that the RAGE overexpressing WM115 cells exhibited mesenchymal-like morphologies compared to the control cells (Meghnani et al., 2014). Based on these results, we suggested that RAGE overexpression in the WM115 cells resulted in a metastatic-like switch of the WM115 cells, where cells switched from a proliferative to a migrative phenotype.

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To further understand the role of RAGE in melanoma tumor progression, we implanted the RAGE and MOCK transfected WM115 cells in SCID mice and compared the growth of resulting tumors. We also analyzed the level of RAGE and its S100 protein ligands in the tumor xenografts.

2. Materials and methods

2.1. Reagents and antibodies

All reagents were of biochemical grade. The antibodies used for Western blots were purchased from the following providers. Antibodies against β-actin (#4970), Akt (#4691), phospho-Akt (Ser473, #4060S), SAPK/JNK (#9258), phospho-SAPK/JNK (#4668, Thr183/Tyr185), p44/42 (#4695) and phospho-p44/42 (#9101, Thr202/Tyr204) were all from Cell Signaling Technology (Danvers, MA). Antibodies against p38 (#ab7952) and phosphorylated p38 (#ab38238) were from Abcam (Cambridge, MA). Anti-S100B (#Z0311) was from DakoCytomation (Denmark), anti-S100A10 (#ab52272) was from Abcam. Rabbit sera directed against, S100A2, S100A4 and S100A6 were a generous gift from Prof. Heizmann (Children's Hospital, Zürich, Switzerland). Anti-S100A8 (#ab92331) and anti-S100A9 (#ab105472) antibodies, recognizing murine S100A8 and S100A9, respectively, were from Abcam. The non-specific murine IgG antibodies were from Innovative Research (Novi, MI). Horseradish peroxidase secondary antibodies specific for the species of the primary antibodies were from Jakson ImmunoResearch Laboratories (West Grove, PA).

The IgG 2A11 producing hybridoma cell line was generated in our laboratory (unpublished results). IgG 2A11 was purified from hybridoma supernatants using a single step chromatography on protein-G sepharose column (GE Healthcare). The purity and integrity of the purified antibody were determined by Coomassie Blue stained SDS PAGE and was estimated to be more than 95%.

2.2. Cell-lines

The generation and characterization of the WM115-MOCK and WM115-RAGE cell lines has been described in a previous publication (Meghnani et al., 2014). The stably transfected cells were maintained in Opti-MEM (Invitrogen) containing 4% FBS (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin and in the presence of 1 mg/ml G418 (WM115-RAGE cells) or 0.5 mg/ml G418 (WM115-MOCK cells).

2.3. Animal studies

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at NDSU and in compliance with the NIH's Principles of Laboratory Animal Care. We used five to six week old female Severe Combined Immunodeficiency (SCID) mice (20–25 g) (Charles River Laboratories, Wilmington, MA) in all our studies.

To compare the growth rate of tumors established from WM115-MOCK and WM115-RAGE cells, we implanted two groups of mice ($n = 8$) subcutaneously, with either WM115-MOCK or WM115-RAGE cells (1×10^6 in 50 μL) under anesthesia. The growth of the tumors was measured every three days with a digital caliper and the tumor volumes were determined using the following equation: $0.52 \times L \times W^2$, where W is the width and L is the length of the tumor (Bremer et al., 2001).

To determine the effect of anti-RAGE antibody treatment on tumor growth, 40 SCID mice were implanted either with WM115-MOCK cells ($n = 16$) or WM115-RAGE cells ($n = 24$) as described above. When the tumor sizes reached 80 mm^3, the mice implanted with WM115-RAGE cells were divided into 3 groups: Mice in group A ($n = 8$) were treated with saline (PBS); mice in group B ($n = 8$) were treated with non-specific murine antibodies; mice in group C ($n = 8$) were treated with IgG 2A11 in PBS (0.5 mg/mouse, in 100 μL) every 5 days, for 30 days (Cruz-Munoz et al., 2009). The mice implanted with WM115-MOCK cells were also separated into two groups: mice in group D ($n = 8$) were treated with murine control antibodies; mice in group E ($n = 8$) were treated with IgG 2A11 in PBS (0.5 mg/mouse) every 5 days, for 30 days. The antibody was administered *via* intraperitoneal injection.

To determine the effect of dacarbazine on the growth of the melanoma tumor xenografts, 32 mice were implanted with WM115-RAGE cells as described above. When the tumor sizes reached 80 mm^3, the mice were randomly assigned to 5 groups. Mice in groups F, G and H ($n = 8$) were treated with dacarbazine at dosages of 50 mg/kg, 25 mg/kg and 12.5 mg/kg, respectively. Mice in group H ($n = 4$) were treated with dacarbazine (25 mg/kg) and IgG 2A11 (0.5 mg in 100 μL of PBS per mouse every 5 days), and mice in group I ($n = 4$) were treated with dacarbazine (12.5 mg/kg) and IgG 2A11 (0.5 mg in 100 μL of PBS per mouse every 5 days). The antibody and dacarbazine were administered through intraperitoneal injection.

To image the tumors in mice, we used the anti-RAGE IgG2A11 antibody labeled with the infra-red fluorescence dye Cy5.5 (Hsiao et al., 2006). A non-specific murine control antibody was used as negative control. Briefly, 16 mice were implanted with wither WM115-RAGE cells (Groups J, K and L, $n = 4$/group) or WM115-MOCK cells (Group M; $n = 4$). When the tumor volumes reached 80 mm^3, the mice implanted with WM115-RAGE cells were separated into three groups. Mice in groups J, K and L were injected in the tail vein with the Cy5.5 labeled RAGE antibody (100 μg in 125 μL), the Cy5.5-labeled mouse control IgG (100 μg in 125 μL) and PBS respectively. Mice in group M ($n = 4$) were injected with Cy5.5 labeled RAGE antibody (100 μg in 125 μL). At 0, 4, 24, and 48 h after injection, the mice were anaesthetized and subjected to near infra-red fluorescence (NIRF) imaging using a Kodak FX Pro Imager (Carestream Health Incorporation, Rochester, NY) (Hsiao et al., 2006). The image acquisition time was set at 1 min. The images were analyzed using the Kodak Digital Science 1D software. The average fluorescence intensities at the region of interest (ROI) were corrected by subtracting the background fluorescence of the adjacent skin.

To study the biodistribution of IgG 2A11 in mice, we used the mice that were used for the imaging studies (Groups J–M). At 48 h after antibody injection, the animals ($n = 3$ from each group of animals) were sacrificed and the tumors and organs (liver, spleen, kidneys, heart, brain, bladder and stomach) were excised, washed with PBS, and subjected to imaging. The average fluorescence intensities were determined by selecting the whole organs as the region of interests. The image acquisition time was set at 1 min.

In all the studies, the health of the animals was observed daily, the tumor size and the body weight of animals were recorded every three days. At the end of the treatment or if the tumor size exceeded 1200 mm^3, the mice were euthanized and the tumor were excised. These tumors were either immediately prepared for immunohistochemistry or snap frozen in liquid nitrogen for RT-PCR and Western blot analysis.

2.4. Western blots

Protein extracts were prepared from cells and tumors using the PARIS kit (Ambion/Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The protein content was determined with the Pierce BCA protein assay kit (Pierce/Thermo Scientific, Rockford, IL). The proteins (40–100 μg) were separated on either 10% or 15% SDS gels and then electro-blotted onto nitrocellulose membranes. Depending of the manufacturer's