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Overexpression of receptor of advanced glycation end products hypersensitizes cells for amyloid beta peptide-induced cell death

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Abstract

Receptor of advanced glycation end products (RAGE) was identified as one of the receptors for amyloid beta peptide (Aβ). There is evidence for controversial functions of RAGE such as a mediator of cell death or differentiation. In this report, we demonstrate that RAGE mediates Aβ toxicity. Transient transfection of RAGE already induced cell death. For further analysis, stable clones of hemagglutinin (HA)-tagged RAGE were selected. Analysis of cellular localization of HA-tagged RAGE protein revealed, in addition to the expected cell surface expression, a novel intracellular localization. Stable RAGE-expressing cells were hypersensitive to nanomolar amounts of Aβ. Only cells expressing RAGE at the cell surface showed hypersensitivity to Aβ.

Keywords: Amyloid beta peptide; Hemagglutinin; RAGE

1. Introduction

The human receptor of advanced glycation end products (RAGE; AGER) is a 404 aa protein. Transmembrane domain is assigned to a 21 aa region, resulting in a large extracellular domain (320 aa) and a small intracellular domain of 41 aa. RAGE belongs to a family of cell surface molecules with immunoglobulin folds. Sequence homologies exist to MUC18, a glycoprotein which also belongs to the immunoglobulin superfamily and to neural cell adhesion molecule (NCAM). Furthermore, the intracellular domain of RAGE is similar to the B cell activation marker CD20 [1]. RAGE is expressed in endothelial cells, in mononuclear phagocytes (monocytes, macrophages, mesangial cells), in neurons, and muscle cells. On the other hand, the expression of RAGE on neurons was refuted by Ref. [2]. During development, RAGE is expressed at a high level in the nervous system [3]. Induced expression is frequently associated with pathological stages such as diabetical endothelial damage and Alzheimer disease [4]. Recently, it was shown that blocking of RAGE could restore wound healing in diabetic mice [5]. Blocking T cell RAGE can inhibit T cell infiltration, which provides the means to interfere with the development of experimental autoimmune encephalomyelitis [6]. The Stern group [1] discovered RAGE as a receptor for advanced glycation end products (AGEs). AGEs can be built by non-enzymatic glycosylation of proteins during aging or diabetes mellitus. Proteins with slow turnover in tissues with low mitotic activity such as in the central nervous system are frequently afflicted by such glycosylation processes. There are additional physiological ligands which bind to RAGE. Amphoterin, a 30 kDa developmental regulated protein is another RAGE ligand [3]. Interestingly, amphoterin stimulates the outgrowth of neurites [3]. Furthermore, the RAGE amphoterin binding can lead to uncontrolled growth of brain tumors and to the induction of metastasis, which might be blocked by interference with the amphoterin/ RAGE signaling [7].

One of the hallmarks of Alzheimer disease is the formation of deposits (plaques) within the brain of patients [8]. Later on, one of the major components of these plaques could be identified as the amyloid beta peptide (Aβ) derived from amyloid precursor protein [9]. Aβ was identified as a neurotoxic and neurotrophic peptide dependent on the concentration [10].

In order to establish a human model for Aβ mediated cell death, we transfected RAGE in cell lines. Here we

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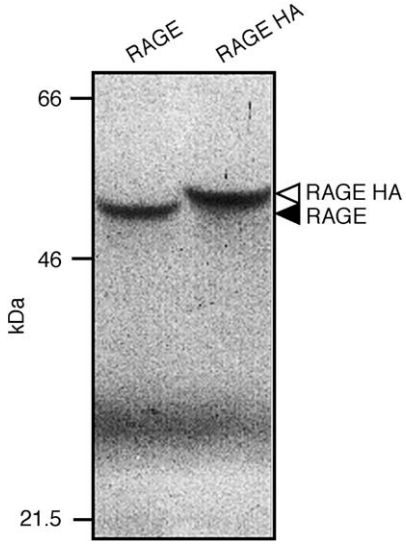

Fig. 1. In vitro translation of RAGE and RAGE HA cDNAs. Expression vectors for RAGE cDNA with or without hemagglutinin tag were submitted to a coupled in vitro transcription and translation reaction. Reaction products were separated on a SDS gel and visualized after blotting using incorporated biotin labels, revealing the expected molecular weights (RAGE, 50 kDa; RAGE HA, 53 kDa) of both products.

report that RAGE is localized in stable transfectants to a high degree in intracellular compartments. Comparing the induction of cell death by Aβ in stable RAGE transfectants, we found that cell surface expression is important for induction of cell death. Cell surface expression of RAGE results in a hypersensitivity against Aβ even in nanomolar doses, which were previously reported to be neuroprotective [11].

2. Material and methods

2.1. Cell culture and treatment

COS-1 cells (American Type Culture Collection, Rockville, MD, USA) and 293 QBI cells were grown in DMEM containing 10% fetal calf serum, 1% penicillin/streptomycin. Cultivation was at 37 °C and 5% CO_2. Treatment was done on 8-chamber slides (Nunc). Cells were plated 24 h before treatment. Cells were treated with Aβ 1–40 (Bachem, Heidelberg) at the concentrations indicated or mock-treated (treated without peptide). Aβ 1–40 was aged for 5 days at 37 °C. Neurotoxicity was assayed before use as described [11,12].

2.2. Vectors, transfection and translation

RAGE cDNA was cloned via PCR using published sequences [1,13]. Expression vector pUT SV1 (pZEO SV1) was obtained from Cayla, Toulouse, France. For immunological detection, the stop codon of RAGE was changed to include a HA tag (GYPYDVPDYA) fused to the C-terminus. Cloning of PCR fragments were done using *Eco*RI sites. Cells were transfected using FuGENE 6 (Roche) according to the protocols provided by the manufacturer. Selection of stable clones was done using 100 μg/ml Zeocin.

Vectors containing RAGE cDNAs were submitted to a coupled in vitro transcription and translation reaction (TNT, Promega). After separation of products on a 8% PAA, SDS gel detection was done after blotting on a PVDF according to the protocols provided by the manufacturer (Transcend, Promega).

2.3. Immunostaining and cell death analysis

Cells were fixed for 2 min in EtOH and for 5 min in 3.7% formaldehyde and immunostained with an anti-HA mAb (Roche; diluted 1:50), detected with an anti-mouse Ab coupled to Cy3 (Jackson Labs, 1:400). X-Gal staining of lacZ transfected cells was done as previously described [11]. Cells were scored dependent on their morphology as dead or alive as described by Ref. [14] using the following criteria: cells alive depict a flat trapezoid morphology, whereas dead cells are small and rounded up. Statistical analysis was

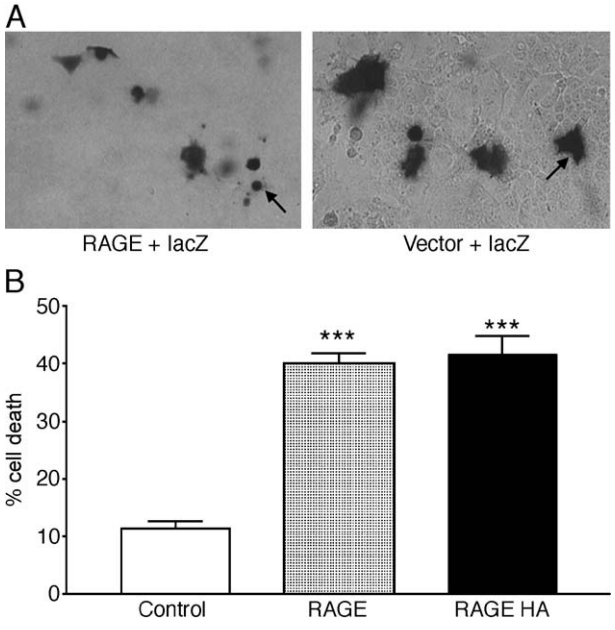

Fig. 2. Transient transfection of RAGE reveals induction of cell death. (A) COS-1 cells were transfected with RAGE and lacZ expression vectors (left panel) or with pUT SV1 and lacZ (right panel). Forty-eight hours after transfection cells were fixed and stained with X-Gal. Transfected dead cells were identified as small round pyknotic cells (arrow in left panel) in comparison to living cells, depicting a flat morphology (arrow in right panel). (B) Quantification of cell death in blue cells reveals a significant amount of cell death in RAGE and RAGE HA tansfectants. Cell death of blue cells was determined 48 h after treatment. Data are shown as the mean of 30 independent determinations. Error is depicted as S.E. (***) Marks significant difference to control ($P < 0.01$).

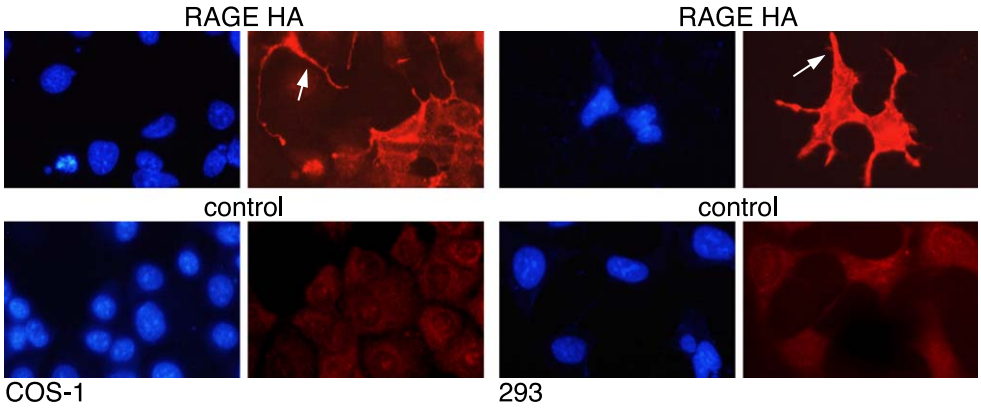

Fig. 3. Membrane localization of RAGE in transient transfectants. Immunostains (red) using anti-hemagglutinin antibodies were performed on RAGE HA-transfected COS-1 cells (left panels) and 293 cells (right panels). Cell nuclei were stained with DAPI (blue). Cells transfected with empty expression vectors (control) were depicted in lower panels. Arrows mark examples of membrane-localized immunoreactivity.

performed with ANOVA followed by Scheffé's post hoc test.

3. Results

To determine the effect of RAGE on cell death, two RAGE expression constructs were prepared; one containing the coding region of human RAGE and a second one containing, in addition to RAGE, an influenza hemagglutinin tag. In order to test if the proteins could be expressed, both constructs were translated in vitro using rabbit reticulocyte extracts. SDS page revealed that both translation products showed the predicted molecular mass (see Fig. 1).

To rule out a biological difference between the tagged and the wild-type RAGE, transient transfectants were analyzed. Cell death was chosen as a read-out. Both wt RAGE and tagged RAGE were transfected in COS-1 cells together with a lacZ expression vector as a marker of transfected cells. Blue cells were scored morphologically for cell death [11]. Surprisingly, the transfection of both tagged and untagged RAGE induced a considerable amount of cell death, in comparison to lacZ transfectants (see Fig. 2). No difference in the induction of cell death was observed between tagged and untagged RAGE. Therefore, tagged

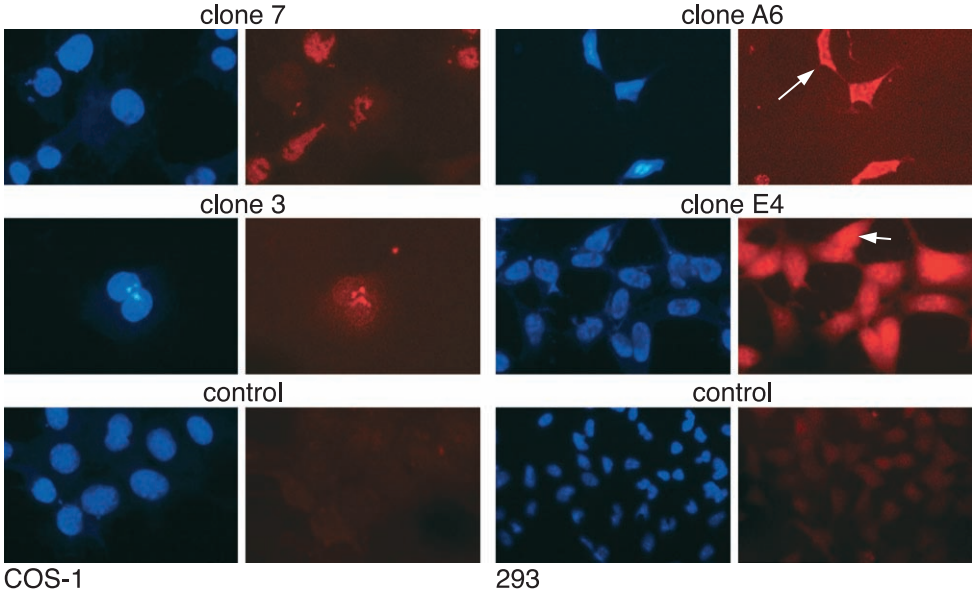

Fig. 4. Localization of RAGE in stable transfectants. Examples of clones of COS-1 and 293 transfectants are depicted. Surprisingly, no membrane localization was detected in many COS-1 clones (see clones 7 and 3, left panels). 293 clone A6 depicted membrane localization (see arrow, upper right panel) but clone E4 depicted an intracellular localization (see arrow, middle right panel). Controls were empty vector transfectants. Staining was done as for Fig. 3 (anti-HA immunoreactivity in red; nuclei in blue).

RAGE was chosen for further analysis, since it offers the advantage of easy immunological detection.

Stable tagged RAGE transfectants were analyzed, both in HEK 293 and in COS-1 cells. Immunostaining with an antibody directed against the HA tag of RAGE revealed a robust protein expression (Fig. 3). Surprisingly, there were two classes of clones, which differed in their localization of RAGE. As expected, there are clones such as A6 (see Fig. 4), showing membrane staining (compare staining of transfectants to control staining depicted in the lower panel). But there are clones where RAGE seemed to be localized intracellularly (see Fig. 4, clone E4). Both, the membrane localized RAGE (clone A6) and the one with pronounced intracellular localization (clone E4) are expressed at comparable levels, as visible in the immunofluorescence. Thus there should be no differences in the potential RAGE receptor availability.

Is there any biological function, which is different between those two clones with different cellular localization of overexpressed RAGE? Both clones were tested for sensitivity to Aβ-mediated cell death (Fig. 5A). The human 293 cells used here offer the advantage of being relatively flat on a culture dish and thus dead cells could be easily identified using morphological criteria. Visual inspection of transfected 293 cells was previously established also as a high throughput screening for apoptosis by Grimm and Leder [14]. Here we followed Grimm's description and identified dead cells as rounded non-flat cells, which is a very conservative estimate, since many of the dead cells might be already in the supernatant and will not be counted. A dose response curve from 0.1 to 10 μM Aβ was analyzed (Fig. 5B). At 10 μM Aβ nearly all cells were dead, independently of the presumed localization of RAGE. In contrast, a striking difference was detected when using a concentration of 1 μM Aβ. Cells with membrane localized RAGE (clone A6) were clearly more sensitive to Aβ than cells with intracellular RAGE (clone E4) or empty vector transfectants (mock). This hypersensitivity to Aβ was also detected at a concentration of 0.1 μM Aβ. Next, we asked if there might be a difference already seen at even lower concentrations. When analyzing a con-

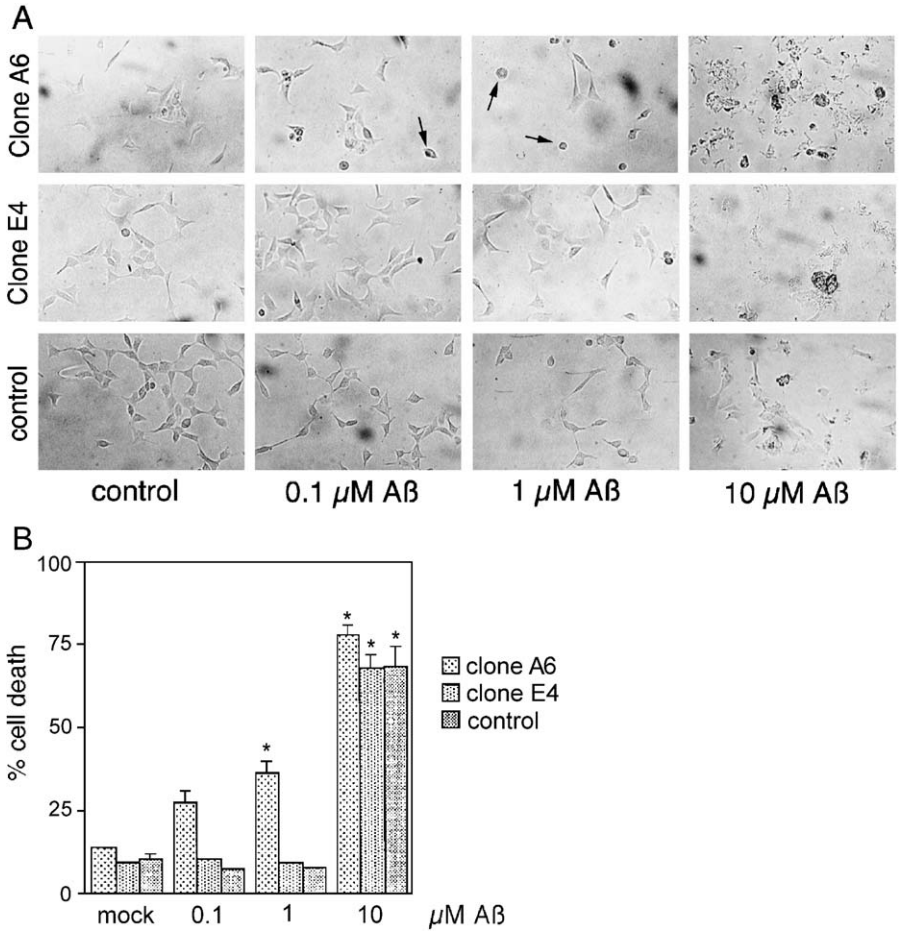

Fig. 5. Cell death analysis of stable RAGE transfectants treated with high doses of Aβ. Two stable clones of 293 cells transfected with RAGE HA were subjected to Aβ treatment and cell death was determined after 24 h. (A) Phase contrast images of clones E4, A6 and empty vector transfectants. Note the increased sensitivity to Aβ toxicity of clone A6 (membrane-localized RAGE). Dead cells are recognized as rounded up (see arrows). (B) Quantification of cell death reveals increased Aβ sensitivity. Data are shown as mean (bar depicts 95% confidence interval) of 10 independent determinations. A star marks significant difference to mock-treated (without peptide) cells ($P < 0.05$). Note that 1 μM induces significant cell death only in clone A6 (membrane-localized RAGE).

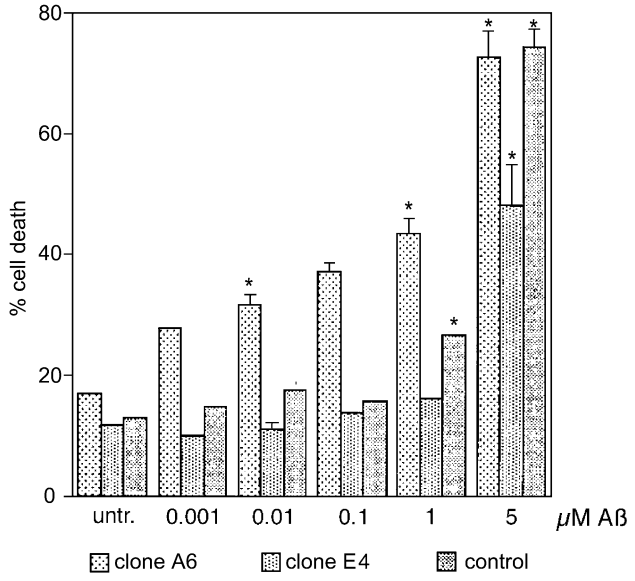

Fig. 6. Cell death analysis of stable RAGE transfectants treated with low doses of Aβ. Data are shown as mean (bar depicts 95% confidence interval) of 10 independent determinations. A star marks significant difference to mock-treated cells (untr.) ($P<0.05$). Note that 0.01 μM already induces significant cell death, but only in clone A6 (membrane-localized RAGE).

centration range spanning from 0.001 to 5 μM Aβ, a hypersensitivity of RAGE transfectants (Fig. 6, clone A6) could already be detected at a concentration of 0.01 μM Aβ. Taken together, surface expression of RAGE induces a vastly increased sensitivity to normally subtoxic Aβ amounts.

4. Discussion

This study demonstrates that RAGE overexpression sensitizes transfected cells to Aβ induced cell death in two model cell lines (COS-1 and HEK 293). Cell death was already induced in transient transfectants. This might explain why the RAGE expressing stable clones could be isolated at a lower frequency in comparison to the lines transfected with the vector without RAGE cDNA. Not only overexpression, but also localization of RAGE is important for cell death induction. Cells with a stable intracellular localization of RAGE could be isolated from both cell lines. As hypothesized from the immunolocalization of tagged RAGE, these cells were resistant to Aβ-induced cell death. We conclude that Aβ-induced cell death of RAGE transfectants is not based on any unspecific sensitivity of cells overexpressing a protein, but is based on RAGE as cell surface membrane receptor of Aβ. No difference in the cell death inducing capability of tagged and untagged RAGE was detected. This might argue for an intracellular domain of RAGE, which is signaling independently of the HA-tag. In transient transfections, we observed a high amount of cell death in comparison to cells transfected with the vector backbone of RAGE expression constructs. This might be due to low amounts of Aβ already present in the culture medium as a consequence of the ubiquitous expression of Aβ precursor protein. There are several intracellular reactions described, which follow the activation of RAGE. Many of the described physiological reactions of RAGE activation evoke proliferation or cell differentiation. Prominent example of RAGE-mediated proliferation is tumor growth [7]. Binding of protein ligand amphoterin to RAGE on cortex neurons induced increased formation of neurites [3,15]. RAGE ligation in embryonic stem cells promoted neuronal differentiation [16]. In neuroblastoma cells differentiated with retinoic acid, RAGE seems to be more important for survival [17]. Soluble AGEs could similarly to Aβ induce migration of monocytes, whereas surface fixed ligands of RAGE could fix the cells to the ligation of the ligand [4]. These different, partly counteracting reactions exemplify, that RAGE activation might only be the first step of a signaling cascade with great divergence. Some further details of the intracellular processes following RAGE activation are already highlighted. For both RAGE-dependent neurite outgrowth and RAGE-dependent NF-κB activation, the cytoplasmic RAGE domain is necessary. Divergence develops through different intracellular interactions with the intracellular RAGE domain. For neurite outgrowth activation of the GTPases Rac and Cdc42 seems to be essential. But for NF-κB activation, the GTPase Ras might be necessary [18]. Which components are involved in RAGE-mediated cell death is currently under investigation. What directs the membrane or intracellular localization of RAGE is currently unknown. This might very well require additional proteins or receptors, which are only available in limiting amounts. Future studies will address the potential of further RAGE interacting proteins.

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