Alpha1-antichymotrypsin induces TNF-α production and NF-κB activation in the murine N9 microglial cell line

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ABSTRACT

Microglia are known to accumulate in senile plaques of Alzheimer’s disease (AD) together with a set of proteins including α1-antichymotrypsin (ACT). To investigate the biological effects of the interaction between ACT and microglia, we examined cytokine production by the murine N9 microglial cell line after ACT treatment. Real-time PCR analysis and specific immunoassays demonstrate that ACT triggers mRNA expression and release of TNF-α by N9 microglial cells. Furthermore, we show that ACT induces a significant increase in NF-κB nuclear translocation. Taken together, these data demonstrate that ACT might contribute to the inflammatory mechanisms present in AD senile plaques.

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Senile plaques of Alzheimer’s disease (AD) brain are characterized by extracellular deposition of beta-amyloid (Aβ) surrounded by dystrophic neurites and activated microglia and astrocytes. The spatial association of Aβ aggregates with complement proteins, pro-inflammatory cytokines and acute-phase proteins, such as α1-antichymotrypsin (ACT) [7], suggests a significant role of inflammation in AD pathogenesis. In fact, Aβ alone might be relatively harmless when inflammation and promoters of Aβ polymerization are absent [12].

ACT is a member of the serine protease inhibitor family termed “serpins” and is not present in normal brain [3,5]. It is highly expressed in astrocytes of both amorphous and classical plaques of AD [2], and can promote the formation of, and is associated with, the neurotoxic Aβ deposition [12]. Besides a regulatory role in the formation of Aβ fibrils, ACT is also able to activate a pro-inflammatory response in astrocytes, including upregulation of IL-1β [8] and monocyte chemoattractant protein-1 (MCP-1) [5] as well as production of nitric oxide through increased expression of the inducible nitric oxide synthase (iNOS) [8].

A number of different stimuli can induce microglial activation, including Aβ1-42 peptide [11], the major constituent of classical plaques. However it is unknown whether ACT itself might activate microglia.

In this study, we investigated whether cytokine production can be induced by interaction between ACT and microglia. In particular, we studied the gene expression and secretion of TNF-α as well as NF-κB activation in a murine microglial cell line (N9), that closely reproduces the inflammatory response of microglial primary cultures [13].

The murine N9 microglial cell line, obtained by immortalization of embryonic brain cultures with the 3RV retrovirus carrying an activated v-myc oncogene [13], was kindly provided by P. Ricciardi-Castagnoli (Centro di CitoFarmacologia, Milan, Italy) and grown in RPMI-1640 medium with 25 mM HEPES and 1-glutamine (Euroclone, Pero, Italy) containing antibiotics, amphotericin B and 10% heat-inactivated fetal bovine serum.

Cells were treated with ACT (Calbiochem, La Jolla, CA, USA) at a concentration of 10, 30 and 40 μg/ml for up to 48 h. Cell culture media were then harvested and analyzed for the presence of TNF-α using a specific enzyme immunoassay kit (R&D Systems, Minneapolis, MN, USA). To exclude the presence of endotoxin in ACT stocks, cells were incubated with ACT in the presence of polymyxin B sulfate (PMX) (Sigma), an antibiotic used for lipopolysaccharide (LPS) neutralization [10].

Total RNA for each condition was isolated using the TRIzol reagent (Invitrogen) protocol and reverse-transcribed with the Ready-To-Go You-Prime First-Strand Beads kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). TNF-α mRNA levels were quantified with an ABI 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA), using a specific TaqMan TNF-α probe (Mm00443258m1, Applied Biosystems). The relative
amount of mRNA was determined by comparison with the "housekeeping" murine β actin probe (Mm00607939s1, Applied Biosystems). Relative TNF-α mRNA levels were calculated as follows: $2^{-\Delta\Delta Ct} = 2^{-\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}}$, where ΔCt equals the TNF-α threshold cycle number (Ct) minus the Ct of β actin.

In order to quantify NF-κB, cells were incubated for 1 h with 40 μg/ml ACT or 100 ng/ml LPS (from Escherichia coli 026.B6, Sigma, St Louis, MO, USA) and then scraped from each flask for nuclear extraction. NF-κB activation was measured in nuclear extracts with NF-κBp65 (total) ELISA kit (BioSource, Nivelles, Belgium).

Statistical evaluation was performed using the Student’s t-test for single comparisons in the time-course study, whereas in case of multiple comparisons statistical significance was tested with one-way ANOVA followed by Bonferroni’s test as calculated by Prism 4.02 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was set at $p < 0.05$. Data are expressed as means ± SD.

As shown in Fig. 1A, untreated cells did not constitutively release TNF-α up to a 48-h incubation, whereas TNF-α production was significantly up-regulated in response to ACT. Dose-response studies demonstrated that the amount of TNF-α released into the culture medium increased with increasing ACT concentrations (Fig. 1B). In particular, TNF-α production was significant with 30 μg/ml ACT (28.15 ± 5.55-fold increase over untreated cells; $p < 0.01$) and 40 μg/ml ACT (36.87 ± 5.55-fold increase over untreated cells; $p < 0.01$), although not as high as with 100 ng/ml LPS (300.17 ± 12.95-fold increase over untreated cells; $p < 0.01$), used as a positive control. Kinetics experiments revealed that TNF-α release in response to 40 μg/ml ACT was already significant after 12 h of incubation (28.85 ± 0.59-fold increase over untreated cells; $p < 0.001$) and increased progressively over time (Fig. 1A).

The specificity of these effects was demonstrated by using PMX, which did not influence ACT-induced stimulation, but almost completely suppressed LPS capability to increase TNF-α production (Fig. 2).

In subsequent experiments, we investigated the expression of TNF-α mRNA through real-time PCR analysis. As shown in Fig. 3, TNF-α mRNA increased after 4 h of stimulation with ACT at the concentration of 30 μg/ml (2.25 ± 0.13-fold increase over untreated cells; $p < 0.001$) and 40 μg/ml (2.47 ± 0.16-fold increase over untreated cells; $p < 0.001$).

We finally studied whether ACT is also able to induce NF-κB nuclear translocation in N9 cells. Incubation for 1 h with 40 μg/ml ACT triggered NF-κB activation (7.79 ± 0.07-fold increase over control; $p < 0.01$) (Fig. 4).

In this study, we demonstrate that ACT is able to induce TNF-α production by murine N9 microglial cells. This effect is regulated at a transcriptional level, as indicated by the increased expression of TNF-α mRNA in ACT-stimulated N9 microglial cells. Specificity of ACT effects was demonstrated using PMX, which did not influence TNF-α accumulation by N9 microglial cells treated with ACT. In these experiments we also show that ACT activates the nuclear transcription factor NF-κB in N9 cells. Since NF-κB is involved in the induction of TNF-α gene expression [6,15], we speculate that NF-κB nuclear translocation triggered by ACT might represent one...
of the molecular mechanisms underlying the inducible expression of TNF-α gene in murine microglia.

The ability of N9 microglia to respond to ACT with TNF-α production suggests a role of ACT in the chronic inflammatory response observed in AD classical plaques. This hypothesis is supported by the presence of TNF-α [18] and ACT [1,14] together with the evidence of microglial activation [17] in classical plaques. At these sites, we suggest that reactive astrocytes producing ACT may induce TNF-α release through their ability to stimulate microglia in a paracrine fashion. TNF-α production triggered by ACT may have manifold consequences in AD pathogenesis, because transgenic mice overexpressing TNF-α exhibit severe inflammation and neurodegeneration [4].

Immunocytochemical studies have demonstrated that ACT is not only present in classical neuritic plaques but also in diffuse plaques [16]. Since diffuse plaques lack of remarkable inflammation, a question raised by our study is the pathophysiological role of ACT at the early stage of senile plaque formation. In this regard it has been suggested that ACT is involved in proteolytic degradation of the amyloid precursor protein into amyloid fibrils [14]. Moreover, the absence of substantial inflammatory response in diffuse plaques containing ACT might be explained by the fact that microglia are not involved in the formation of diffuse plaques [9].

In conclusion, our results indicate that ACT interaction with N9 microglial cells might contribute to inflammation in neuritic plaques, also through TNF-α production. These findings suggest that inhibition of ACT deposition in senile plaques could significantly reduce detrimental consequences of microglial activation in AD.

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