Activation of Microglia Acidifies Lysosomes and Leads to Degradation of Alzheimer Amyloid Fibrils

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Microglia are the main immune cells of the brain, and under some circumstances they can play an important role in removal of fibrillar Alzheimer amyloid β peptide (fAβ). Primary mouse microglia can internalize fAβ, but they do not degrade it efficiently. We compared the level of lysosomal proteases in microglia and J774 macrophages, which can degrade fAβ efficiently, and we found that microglia actually contain higher levels of many lysosomal proteases than macrophages. However, the microglial lysosomes are less acidic (average pH of ~6), reducing the activity of lysosomal enzymes in the cells. Proinflammatory treatments with macrophage colony-stimulating factor (MCSF) or interleukin-6 acidify the lysosomes of microglia and enable them to degrade fAβ. After treatment with MCSF, the pH of microglial lysosomes is similar to J774 macrophages (pH of ~5), and the MCSF-induced acidification can be partially reversed upon treatment with an inhibitor of protein kinase A or with an anion transport inhibitor. Microglia also degrade fAβ if lysosomes are acidified by an ammonia pulse-wash or by treatment with forskolin, which activates protein kinase A. Our results indicate that regulated lysosomal acidification can potentiate fAβ degradation by microglia.

INTRODUCTION

Extracellular senile plaques containing fibrillar forms of amyloid-β peptide (Aβ) are observed in brains of Alzheimer’s disease (AD) patients (Selkoe, 2001). The Aβ peptide is a 39–42 amino acid fragment derived by proteolytic processing from a larger membrane-spanning glycoprotein called β-amyloid precursor protein (βAPP). Aβ peptides form fibrillar β-pleated sheet structures (fAβ) that are the major component of senile plaques. Although there may be multiple initial causes of AD, inherited mutations in βAPP that lead to excess Aβ production are strongly associated with familial early onset AD (Selkoe, 2001), indicating that excess Aβ can be causative for AD. Furthermore, treatments that lead to clearance of fAβ in mouse models of AD have been reported to be associated with some reversal of behavioral impairments (Morgan et al., 2000; Hartman et al., 2005).

Net accumulation of fAβ is related to the rate of production of Aβ and to its rate of degradation. As the main immune cells of the brain, microglia would be expected to play a role in clearing extracellular debris similar to a function carried out elsewhere by macrophages, a closely related cell type. Indeed, under some circumstances it has been shown that microglia can degrade fAβ. Immunotherapy against Aβ leads to a striking removal of amyloid plaques in βAPP transgenic mice (Schenk et al., 1999), and there is good evidence that microglia are responsible for plaque clearance (Bard et al., 2000). In humans, microglia also are frequently found in regions surrounding amyloid plaques in AD brains, and Aβ was found in microglia in a human patient immunized against Aβ (Nicoli et al., 2003). The regions surrounding such microglia were devoid of extracellular plaques, suggesting that the microglia had removed plaque material.

Primary mouse microglia in tissue culture do not degrade fAβ even though they internalize it rapidly by receptor-mediated uptake and deliver it to lysosomes that can degrade other endocytosed proteins in the same organelles (Paresce et al., 1997; Chung et al., 1999). Furthermore, primary microglia in tissue culture do not degrade fAβ when it is coated with anti-Aβ antibodies with or without the addition of complement components (Brazil et al., 2000), although microglia in brain slices could digest some plaques (Bard et al., 2000). These findings indicate that binding via immune receptors is not sufficient to trigger degradation of internalized fAβ, but a more complex activation mechanism is needed to facilitate degradation. This is also supported by studies in APP transgenic mice in which immunotherapy effectively reduced fAβ deposits even in an FcγR knockout background (Das et al., 2003).

Primary mouse microglia in tissue culture actually release macromolecular fAβ that had been internalized (Chung et al., 1999). This suggests that microglia in a nonactivated state would be unable to clear fAβ from the brain, which may account, in part, for the accumulation of fAβ. Consistent with the release observed in tissue culture, reports based on post-mortem examination of the relationship between microglia and
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In Vitro Protease Activity Assay

Lysosomal proteases were assayed as described previously (Tyynela et al., 2000; Awano et al., 2006).

Lysosomal pH Measurement

The measurement of lysosomal/endosomal pH by confocal microscopy (Presley et al., 1993; Dunn et al., 1994) is based on the use of the ratio of the pH-sensitive fluorescent dextran to pH-insensitive rhodamine fluorescence. Cells were incubated for 16 h with 5 ng/ml dextran conjugated to both fluorescein and rhodamine (70,000 mol. wt.; Invitrogen) in complete growth media. Cells were washed thoroughly in complete media, and then they were incubated for another 4 h to chase the dextran to lysosomes. The cells were then washed with medium 2 (150 mM NaCl, 20 mM HEPES, pH 7.4, 1 mM CaCl₂, 5 mM KCl, and 1mM MgCl₂) and kept in medium 2 for 20 min at 37°C in air. Cells were then taken for confocal imaging at 37°C. Confocal images were collected on a LSM510 laser scanning confocal unit (Carl Zeiss, Thornwood, NY) attached to an Axiovert 100 M inverted microscope (Carl Zeiss) with a 63× numerical aperture 1.4 plan Apochromat objective (Carl Zeiss). Excitation on the LSM510 laser confocal microscope was with 25-mW Argon laser emitting 488 nm and a 10-mW helium/neon laser emitting at 543 nm. Emissions were collected using a 500–530-nm band pass filter to collect fluorescein emission and a 560–615-nm band pass filter to collect rhodamine emission. Typically, 0.3- to 0.5-μm vertical steps were used with axial resolution ~1.0 μm. Using image processing techniques developed previously (Dunn et al., 1994), lysosome fluorescence was quantified, and the ratio of fluorescein to rhodamine fluorescence was determined for each individual lysosome. Images were analyzed using MetaMorph image processing software (Molecular Devices). For all experimental sets, cross-talk of the fluorophores was negligible. Calibration curves were generated after fixing and equilibrating the fluorescein/rhodamine–dextran–loaded cells to a range of pH buffers. Although the fluorescein/ rhodamine ratio was sensitive to differential photobleaching of fluorescein and rhodamine fluorescence, the effects of differential photobleaching were minimized by 1) attenuating the laser intensity, 2) minimizing the cells exposure to the lasers, and 3) rigidly adhering to a standard protocol for image collection for both living cells and pH-standardized fixed cells. Consequently, the effect of photobleaching on fluorescein/rhodamine fluorescence was negligible and consistent for all the images.

Degradation of Cy3-fAβ by Microglia after Artificial Lysosomal Acidification

For artificial lysosomal acidification, we used an ammonium chloride pulse-wash technique (Okkuma and Poole, 1978). Cells were washed with medium 2, and then they were incubated for 30 min in medium 2 containing 25 mM ammonium chloride. Cells were washed thoroughly and incubated in medium 2 for another 60 min. After this incubation, cells were transferred to complete culture medium. For Cy5-fAβ degradation with artificial acidification, the cells were first loaded with Cy3-fAβ, and then they were subjected to ammonium chloride pulse-wash at the 1st, 6th, 24th, and 48th h during the 72-h chase period. After the chase period, the cells were imaged using a Leica epifluorescence microscope with a cooled CCD camera (Micromax 521BFT; Princeton Scientific Instruments, Monmouth Junction, NJ, and Roper Scientific, Trenton, NJ), and the Cy3 fluorescence power was quantified using MetaMorph Imaging System software (Molecular Devices).

RESULTS

In Vitro Assay of Lysosomal Proteases

From tissue culture studies, we know that J774 macrophages can degrade the same fAβ that is not digested by microglia (Majumdar et al., 2007). To understand the reason for this difference, we compared properties of microglia and macrophages. First, we compared the levels of key lysosomal proteases in microglia and in J774 cells. As shown in Table 1, most of the lysosomal proteases tested actually showed a higher specific activity in microglial extracts than in extracts from macrophages. Thus, lack of proteases is not the primary cause of poor lysosomal hydrolysis of fAβ by microglia.

Measurement of Lysosomal pH in Live Cells

The in vitro assays of lysosomal enzyme activity were carried out at acidic pH. In cells, the activity of lysosomal proteases is also dependent upon the pH of the lysosomes. Most lysosomal proteases have acidic pH optima, and many are converted from proenzymes to their active form in the acidic lumen of lysosomes. Thus, the pH of lysosomes can greatly influence their proteolytic power. We compared the lysosomal pH of...
Degradation of fA and deliver it to late endosomes and lysosomes (Chung et al., 2007). Because microglia can degrade fA under some conditions, we examined the effects of microglial activation by proinflammatory treatments. It has been suggested that there is a continuum of microglial activation rather than a single activated state (Town et al., 2005), so we tested a variety of agents that might increase microglia degradation of fA. We had shown previously (Majumdar et al., 2007) that after radiolabeled treatment of microglia with or without various proinflammatory treatments increased the ability of microglia to degrade fA, and MCSF had the greatest effect. The MCSF-treated microglia were almost as efficient in degrading fA as J774 macrophage-like cells were found to be in a similar assay (Majumdar et al., 2007). To confirm that the loss of cell-associated fluorescence was due to degradation as opposed to release of macromolecular fA, we examined the properties of material released from the cells. We had shown previously (Majumdar et al., 2007) that after 125I-fA was taken up by J774 cells the radiolabeled material was released mainly as trichloroacetic acid (TCA)-soluble (i.e., low-molecular-weight) degradation products. We verified that >70% of the radiolabeled material released from MCSF-treated microglia after uptake of 125I-fA was also TCA soluble (data not shown). In vitro measurements of lysosomal enzyme ac-

### Table 1. Lysosomal enzyme activity in cell lysates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Macrophage activity</th>
<th>Microglia activity</th>
<th>Microglia activity/macrophage activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tripeptidyl peptidase (TPP) I</td>
<td>50</td>
<td>238</td>
<td>4.74</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>56</td>
<td>80</td>
<td>1.44</td>
</tr>
<tr>
<td>Dipептидил пептидаза (DPP) I</td>
<td>1915</td>
<td>7242</td>
<td>3.78</td>
</tr>
<tr>
<td>Cathepsin H</td>
<td>563</td>
<td>472</td>
<td>0.84</td>
</tr>
<tr>
<td>DPP II</td>
<td>16</td>
<td>79</td>
<td>5.05</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>51</td>
<td>147</td>
<td>2.88</td>
</tr>
</tbody>
</table>

The assay media were buffered as follows: cathepsin D, pH 3.5; TPP I, pH 4.5; cathepsin B, DPP I and DPP II, pH 5.5; and cathepsin H, pH 6.8.

微小胶体和巨噬细胞能通过荧光比率成像的方法识别出晚期吞噬体和溶酶体中的抗原体，抗原体含有胞吞的荧光素-荧光素-偶联的葡萄糖-纤维素（Dunn and Maxfield, 2003）。因为荧光素-荧光素-偶联的葡萄糖-纤维素的荧光在酸性pH下会降低，而在微胶体中pH值接近5.0，所以微胶体的溶酶体荧光是pH依赖的（Dunn and Maxfield, 2003）。如图1A所示，巨噬细胞中平均溶酶体pH值小于5.0，而微胶体中微胶体的溶酶体pH值约为5.9。这种pH值的差异在溶酶体中可能有助于微胶体的降解。

### Degradation of fA by Activated Macrophage-like Microglia

因为微小胶体可以降解fA，所以在某些条件下，我们研究了微小胶体的活性。我们测试了各种agents，它们可以使微小胶体的活性增大大于微小胶体中的微小胶体的活性。它已经被发现，这是一个微小胶体活性的连续谱而不是一个单一激活的状态（Town et al., 2005），所以我们可以测试各种agents，它们可能增加微小胶体的活性。它已经被发现，这是一个微小胶体活性的连续谱而不是一个单一激活的状态（Town et al., 2005），所以我们可以测试各种agents，它们可能增加微小胶体的活性。我们监测了细胞表面表达的CD11b和MHC II在原代微小胶体中的微小胶体作为一个激活的向一个细胞系的转变（Santambrogio et al., 2001; Monsonego and Weiner, 2003）。IL-6也参与了在各种细胞表面蛋白中表达的微小胶体的微小胶体的向微小胶体的转变（Chomarat et al., 2005）。LPS被测试是因为它是一个强烈的炎症介质，它已经被报道，它可以导致在APP转基因小鼠中产生大量细胞（Herber et al., 2004）。我们监测了细胞表面表达的CD11b和MHC II在原代微小胶体中的微小胶体作为一个激活的向一个细胞系的转变（Santambrogio et al., 2001）。我们也监测了细胞表面表达的CD45作为微小胶体活性的一个指标（Herber et al., 2006）。在图2中，治疗用各种炎症介质反应物导致增加，以增加在表面表达的CD11b，MHC II和CD45，这表示微小胶体被激活后向微小胶体的转变。

随着表达于细胞表面的标记阴，这些炎症介质的刺激物可以降低微小胶体的pH值的微小胶体的溶酶体（Figure 1B）。这种最大改变是看到了与MCSF治疗的，它带来了溶酶体的pH值并且被报告为等同于如图1B所示的J774溶酶体。

如果微小胶体的酸化是基础的，那么无酵母降解的fA，则应该可以通过这些治疗。我们使用了一个fA降解的 assay，其中微小胶体采取fA标记有荧光染料Cy3，并且它用于晚期吞噬体和溶酶体（Chung et al., 1999）。当胞内Cy3-fA被降解，荧光的降低来自细胞，而且这种降解的速率是通过测量标记的fA来测量的。我们测量了微小胶体和巨噬细胞的溶酶体pH值（Figure 1A）和微小胶体的频率分布。如图1B所示，J774巨噬细胞和微小胶体的频率分布在不同条件下差异显著（Majumdar et al., 2007）。
The lysosomal pH dropped from 5.9 to 5.3 when ammonia transiently acidifies the lysosomes (Figure 4A). When ammonium chloride is removed from the medium, the efflux of acidic organelles (Ohkuma and Poole, 1978) occurs. When ammonia acidifies the lysosomes, an accumulation of ammonia in lysosomes (Jentsch et al., 2002) can reduce the inside positive electrical potential that is generated by the V-ATPase, allowing greater acidification of organelles. The form of the Cl⁻ channel that is on the lysosomes of microglia is not known, but chloride channels are found on the lysosomes of many cell types, and they are known to regulate the pH of endosomes and lysosomes (Jentsch et al., 2004).

Several types of chloride channel can be activated by phosphorylation by PKA (Bae and Verkman, 1990), so we...
examined the effects of activators of PKA and inhibitors of anion channels on lysosomal pH in microglia. When cellular cAMP levels were elevated by treatment of cells with forskolin, a significant acidification of lysosomes was seen within 45 min, and this acidification was blocked by the anion transport inhibitor DIDS (Figure 5A). We tested whether forskolin treatment could increase the degradation of fAβ by microglial cells. Treatment of microglia with forskolin for the entire 72-h degradation assay caused a loss of cells, so we used a protocol in which cells were treated with forskolin for 45 min during the 1st, 6th, 24th, and 48th h of a 72-h degradation assay. As shown in Figure 5B, forskolin treatment caused a more effective degradation of fAβ compared to untreated cells. Long-term treatment with DIDS was also toxic to the cells, so we could not test the effect of treatment with DIDS on degradation of fAβ.

The signal transduction pathways activated by MCSF in microglia are not well characterized, but in monocytes it is known that increased cAMP is associated with the MCSF-mediated differentiation of monocytes toward macrophages (Wilson et al., 2005). It is also known that treatment of macrophages with inflammatory cytokines can raise cAMP levels (Aronoff et al., 2005). We tested the effects of DIDS and inhibitors of PKA on the lysosomal pH of MCSF-treated cells. The cells were pretreated with MCSF for 5 days as in the other experiments reported here. As shown in Figure 6A, a 45-min treatment of cells with KT5720, an inhibitor of PKA, significantly increased the lysosomal pH in MCSF-treated cells. Treatment of MCSF-treated cells with DIDS for 1 h caused a similar increase in the lysosomal pH (Figure 6B). These results suggest that activation of PKA and chloride channel activity play an important role in microglial lysosomal pH regulation under inflammatory stimulation.

DISCUSSION

Immunotherapy against Aβ leads to a striking removal of amyloid plaques in APP transgenic mice (Schenk et al., 1999), and there is good evidence that microglia play an important role in this clearance (Bard et al., 2000). However, the mechanisms by which immunization promotes clearance and
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Figure 6. Lysosomal pH measurements in activated microglia in presence of PKA inhibitor and chloride channel blocker. Lysosomal pH was measured in treated and untreated microglia. For measurements with PKA inhibitor, KT 5720 at 10 μM was added to MCSF-treated microglia, and pH was measured 45 min after addition. For measurements in the presence of the chloride channel blocker DIDS at 1 mM was added to MCSF-treated microglia, and lysosomal pH was measured 60 min after the addition of DIDS. (A) Frequency distribution of lysosomal pH in MCSF-treated microglia (green curve), untreated microglia (red curve), and KT 5720-treated activated microglia (blue curve). (B) Frequency distribution of lysosomal pH in MCSF-treated activated microglia (green curve), untreated microglia (red curve), and DIDS-treated activated microglia (black curve). Lysosomal pH measurements were obtained from at least 750 lysosomes from three different fields. The average pH values from three different experiments from three different days are shown. Error bars represent the SEM.

degradation by microglia are uncertain. Recent studies have shown that activation of microglia in the absence of antibodies can also lead to the clearance of Aβ plaques in a mouse model of AD (Frenkel et al., 2005). This indicates that antibodies to Aβ are not necessary for clearance of plaques, but it has been unclear how microglial activation or inflammatory stimuli can lead to improved clearance.

The data presented herein show that increased acidification of lysosomes may be the key factor that determines the ability of microglia to degrade fAβ. Microglia constitutively express a scavenger receptor, SRA, that can mediate the binding and uptake of fAβ (El Khoury et al., 1996; Paresce et al., 1996). As shown here, microglia have sufficient levels of lysosomal proteases to degrade proteins effectively; moreover, the levels of most proteases are higher in microglia than in J774 cells, which can digest fAβ efficiently. However, microglia have weakly acidic lysosomes, which would decrease the activity of many lysosomal proteases and could lead to the inability to degrade fAβ effectively. Treatment with inflammatory agents acidifies microglial lysosomes and makes them able to degrade fAβ. Although treatment with inflammatory agents can cause many changes in microglia, it is striking that a pulse-wash treatment with a weak base that transiently acidifies lysosomes also facilitates significant degradation of fAβ. This suggests that lysosomal acidification is the key event that is required for fAβ degradation by microglia. This phenomenon of increased proteolytic power as a consequence of lysosomal acidification was also described in dendritic cells during their maturation (Trombetta et al., 2003).

In the assays of fAβ degradation with the ammonia pulse-wash or the forskolin treatment, we could only transiently acidify the lysosomes. In our experiments, the lysosomes were acidified four times for about an hour each time during a 72-h degradation assay. Nevertheless, we were able to observe ~40% degradation of fAβ as a consequence of these treatments. We typically observe <40% degradation in a 4-h chase even with macrophage cells (data not shown). It is possible that some key proteolytic enzymes are converted from the proenzyme to the active enzyme during the periods of high acidity and that these enzymes remain active during periods of reduced acidity in the microglial lysosomes.

We explored the mechanisms of lysosomal acidification in microglia. Several signal transduction pathways are activated in cells in response to MCSF treatment (Pixley and Stanley, 2004). Activation of PKA by forskolin caused a drop in lysosomal pH similar to that seen in MCSF-treated cells. The acidification caused by forskolin was inhibited by treatment with DIDS, suggesting that anion transport was required for the acidification. These results would be consistent with activation of chloride channels downstream of PKA activation as a mechanism to lower the lysosomal pH in microglia. The lysosomal acidification in MCSF-treated microglia was partially reversed by inhibition of PKA or by treatment with DIDS, again indicating that PKA-dependent activation of chloride channels is important for the regulation of lysosomal acidification of microglia during inflammatory activation. Further work will be required to analyze the signaling pathways involved and to identify the regulated chloride channels on lysosomes of microglia.

There is evidence that immunization of AD model mice against Aβ leads to some reversal of behavioral impairments in addition to clearing amyloid deposits (Morgan et al., 2000; Hartman et al., 2005), and this suggests that this type of therapy might be beneficial. A major limitation is that inflammatory responses to immunization may be difficult to control, and a human trial of Aβ immunization had to be discontinued due to development of meningoencephalitis in some patients (Nicoll et al., 2003).

It is possible that treatments that cause a specific type of microglial activation, leading to increased lysosomal acidification, may be especially beneficial in promoting clearance of plaques. It should be noted that not all types of microglial activation are equally effective. We found that treatment with MCSF, which causes microglia to express macrophage-like surface proteins, was especially effective at lowering lysosomal pH and activating the degradation of fAβ. In contrast, it has been reported that interaction with CD40 ligand can induce microglia to become more like antigen-presenting cells with decreased ability to phagocytose Aβ (Townsend et al., 2005). Another report showed that activation of microglia via the IL-1 pathway might not be important for plaque clearance because IL-1 receptor knockout mouse cleared fAβ deposits after receiving immunotherapy (Das et al., 2006). Other factors may also modulate the ability of microglia to internalize and degrade amyloid plaques. A recent study found that only bone marrow derived microglia
and not resident microglia were capable of clearing fAb deposits from the brains of AD model mice (Simard et al., 2006).

In summary, we show that unactivated microglia do not degrade fAb because they have weakly acidic lysosomes, and lysosomal acidification is a key downstream event leading to fAb degradation by activated microglia. This may also be a key to the effectiveness of therapies to promote clearance of amyloid plaques by microglia, and treatments that acidify microglial lysosomes while minimizing other inflammatory reactions may be particularly beneficial.

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