Proinflammatory cytokines released from microglia inhibit gap junctions in astrocytes: potentiation by β-amyloid

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ABSTRACT

Brain inflammation is characterized by a reactive gliosis involving the activation of astrocytes and microglia. This process, common to many brain injuries and diseases, underlies important phenotypic changes in these two glial cell types. One characteristic feature of astrocytes is their high level of intercellular communication mediated by gap junctions. Previously, we have reported that astrocyte gap junctional communication (AGJC) and the expression of connexin 43 (Cx43), the main constitutive protein of gap junctions, are inhibited in microglia (MG)-astrocyte cocultures. Here, we report that bacterial lipopolysaccharide activation of microglia increases their inhibitory effect on Cx43 expression and AGJC. This inhibition is mimicked by treating astrocyte cultures with conditioned medium harvested from activated microglia. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were identified as being the main factors responsible for this conditioned medium-mediated activity. Interestingly, an inflammatory response characterized by MG activation and reactive astrocytes occurs in Alzheimer’s disease, at sites of β-amyloid (Aβ) deposits. We found that this peptide potentiates the inhibitory effect of a conditioned medium diluted at a concentration that is not effective per se. This potentiation is prevented by treating astrocytes with specific blockers of IL-1β and TNF-α activities. Thus, the suppression of communication between astrocytes, induced by activated MG could contribute to the proposed role of reactive gliosis in this neurodegenerative disease.

Key words: glial cells • inflammation • Cx43 • brain macrophages

In pathological situations leading to neuronal insults, including neurodegenerative diseases, brain glial cells undergo complex phenotypic changes referred as reactive gliosis. This glial response involves microglial cells (MG) and astrocytes that acquire an activated phenotype characterized by specific molecular and cellular features (1–3). Depending on the disease, gliosis can be seen as a preventing or deleterious event for neuronal survival. Consequently, to gain
insight into the role played by glial cells in brain function and diseases, it is important to understand whether and how their interaction can affect intrinsic properties which contribute to glial functions. Among them, a characteristic feature of astrocytes is their high level of gap junctions that provides a direct pathway for intercellular communication between glia (4). Indeed, astrocytes represent the main brain cell population that expresses a large amount of connexins (Cx), the molecular constituents of gap junction channels. These junctions are formed by closely packed intercellular channels constituted by hexameric rings of Cx aligned head-to-head between adjacent cells that allow direct cell-to-cell exchanges. Although, in the mammalian brain, at least 8 different Cxs have been identified, astrocyte gap junctions are mainly composed of Cx43 in vitro and also of Cx30 in situ (5). Because these channels are permeable to ions and to signaling molecules (second messengers, neurotransmitters and energetic compounds), an alteration of astrocyte gap junctional communication (AGJC) may directly interfere with the well-known neuroprotective role of astrocytes (6, 7). Interestingly, the permeability of astrocytes gap junction channels is controlled by number of endogenous compounds released by neurons, as well as glial cells (5). Furthermore, changes in astrocyte AGJC and/or Cxs expression have been associated with several brain inflammatory situations and pathologies that may have implications in neuronal survival, especially in the context of neurodegenerative diseases (5, 8). Thus, alteration of astrocyte gap junction properties may account for a glial contribution to neurodegenerative processes, including Alzheimer's disease (AD). This would be of particular interest for the understanding of this pathology because sites of β-amyloid (Aβ) deposits, one of the molecular hallmarks in AD, are associated with reactive astrocytes and MG that display activated phenotypes.

Previously, we have demonstrated that astrocyte gap junctions are regulated by cultured MG, suggesting that they make a target for astrocyte-MG interaction (9). Accordingly, the present study was undertaken to determine whether astrocyte gap junctions are affected by the activation of MG and to investigate the potential role of Aβ in the regulation of gap junction-induced intercellular communication between astrocytes. Here, we report that activated MG inhibit AGJC and Cx43 expression through the production of identified proinflammatory cytokines and that the action of these released factors is enhanced in the presence of the active fragment of the Aβ peptide.

MATERIALS AND METHODS

All experiments have been carried out in accordance with the European Community Council Directives of November 24th 1986 (86/609/EEC), and all efforts have been made to minimize the number of animals used and their suffering.

Cell cultures

Astrocytes and mixed cultures

Primary astrocyte cultures were prepared from the cortex of newborn (1–2 days) OF1 mice (Charles River, L’Arbresle, France), as described previously (10). Briefly, cells were grown to confluence in DMEM (Sigma-Aldrich, St. Louis, MO), supplemented with penicillin (5 U/ml), streptomycin (5 µg/ml) and 10% FCS (Hyclone, Logan, UT). On day 8, cytosine arabinoside (5 µM) was added to the culture medium for 48 h. The medium was changed twice a week, and
cells were used between 21 and 28 days of culture. Mixed cultures of astrocytes and MG were obtained using the same protocol, but by omitting the cytosine arabinoside treatment.

**Microglial cells**

Cerebral hemispheres were dissected from newborn mice after removal of meninges. After dissociation, cells were seeded into 10-mm culture dishes (NUNC, Polylabo, Strasbourg, France) at 3 × 10^6 cells/10 ml in DMEM containing 10% heat-inactivated FCS (Abcys, Paris, France), as described (11). Medium was changed at days 1 and 3, and cells were collected at day 10 by shaking culture dishes to detach cells adhering to the astrocyte monolayer. The collected population resulted in more than 98% of cells bearing the Mac-1 antigen, a specific marker of mononuclear cells. Freshly collected MG were seeded in DMEM containing 5% FCS and treated with (activated MG) or without (MG) 10 ng/ml of lipopolysaccharide (LPS, Sigma) for 6 h. After this treatment, LPS medium was replaced by fresh medium and MG or activated MG were then scraped off the plastic dish and distributed at the density of 10^6 cells per astrocyte culture dish, for 24 h. On the other hand, the resulting supernatants from MG or activated MG (1.7 × 10^6 cells/ml) were collected and constitute conditioned medium harvested from nontreated (CM) or activated MG (CM*). Finally, CM and CM* were filtered (0.22 µm) and stored at −20°C before use in ELISA or AGJC assays.

**Scrape loading/dye transfer technique**

Experiments were performed as described previously (9, 10) at room temperature by incubating cells for 10 min in HEPES-buffered salt solution containing (in mM): 140 NaCl, 5.5 KCl, 1.8 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, at pH 7.35. Cells were then washed in calcium-free HEPES solution for 1 min and scrape loading/dye transfer technique (SL/DT) was achieved in the same calcium-free solution containing Lucifer yellow CH (LY, 1 mg/ml). After 1 min, cells were washed with the HEPES solution, and LY loaded in the cells was allowed to diffuse through gap junction channels during 8 min. Digitalized images were taken and data were quantified. In all experiments, the fluorescence area of the first row of cells initially loaded, as measured in the presence of the gap junction channel inhibitor carbenoxolone (100 µM, 10 min), was subtracted from the total fluorescence area. To exclude the possibility of LY loading in MG through open Cx43 hemichannels and/or P2X7 purinergic receptors, we exposed confluent MG cultures to LY for 1 min (1 mg/ml in calcium-free solution). The percentages of the fluorescence area compared with the surface of the total microscopic field (four per experiment) were 1.7 ± 0.7% in the absence of LPS treatment, 1.6 ± 0.6% after 6 h of LPS (10 ng/ml) exposure followed by 24 h wash and 4.2 ± 0.9% after 24 h of LPS (10 ng/ml) exposure, (n=5). These numbers indicate that the amount of LY uptake in MG cannot account for the observations reported presently.

**Western blot analysis**

Cells were collected in a small volume of PBS containing orthovanadate (1 mM), β-glycerophosphate (10 mM), complete miniprotease inhibitor (Roche Diagnostics, Basel, Switzerland) and boiled 5 min with Laemmli medium before sonication. Protein concentration was determined with the Bradford method, using BSA as a standard. Proteins were separated by electrophoresis on 10% polyacrylamide gels and transferred onto nitrocellulose. Membranes
were saturated with 5% fat-free dried milk in triphosphate buffer solution and exposed overnight to mouse anti-Cx43 mAb (IgG1, 1:250, BD Bioscience, San José, CA) at 4°C. They were then washed and exposed to peroxidase-conjugated goat anti-mouse IgG (1:2500, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized with the chemiluminescence detection kit (ECL, Amersham, Piscataway, NJ). Finally, blots were also exposed to mouse monoclonal anti-α–tubulin antibody (MW 55 kDa, 1/20,000, Amersham) combined with the above secondary antibody. Semiquantitative densitometry analysis was performed after scanning the bands, with image-analysis software (NIH Image, Scion Corp., Frederick, MD).

**Immunofluorescence and confocal microscopy**

Cells grown on coverslips were fixed at room temperature with 2% paraformaldehyde for 30 min, washed 3 times with PBS and incubated 30 min in PBS containing 10% goat serum (Zymed, San Francisco, CA) and 0.1% Triton-X100. Cells were then incubated overnight at 4°C with mouse anti-GFAP mAb (IgG1, 1:500, ICN Chemicals) or Alexa Fluor 488-conjugated isolectin GS-IB4 (1/100; Molecular Probes, Eugene, OR) to identify astrocytes and MG, respectively. Rabbit anti-Cx43 Ab (1:400, Zymed) or mouse anti-Cx43 mAb (IgG1, 1:400, BD Biosciences) were used when colabeling was performed. After three washes, cells were incubated for 2 h at room temperature with the appropriate secondary Ab, including TRITC-conjugated goat anti-mouse IgG (1/200) and FITC-conjugated goat anti-rabbit IgG (1/500, Southern Biotech, Birmingham, AL). After several washes, coverslips were mounted in Fluoromount and examined by epifluorescence. To determine the astrocyte/MG ratio, cells immunostained for GFAP (red) and isolectin GS-IB4 (green) were examined at ×20 magnification and 15 field images per coverslip for each fluorochrome were acquired with a CCD camera and analyzed with an image analyzer software (Lucia-Nikon, Tokyo, Japan). To quantify Cx43 expression in astrocytes submitted to different treatments, fluorescent Cx43 labeling was examined with a ×20 objective and 20 field images per coverslip were captured. In each field, the number of Cx43 immunoreactive dots was counted using the image analysis software. Alternatively, a confocal laser-scanning microscope (Leica TBCS SP2, Wetzlar, Germany) was used to visualize double immunostainings with a ×63 objective; stacks of consecutive confocal images taken at 500-nm intervals were acquired sequentially with two lasers (argon 488 nm and helium/neon 543 nm) and Z projections were reconstructed using Leica Confocal Software.

**Detection of IL-1β and TNF-α**

ELISA cytokine titrations were performed with conditioned media harvested from MG cultures. Amounts of IL-1β and TNF-α were determined in supernatants by means of specific mouse cytokine ELISA sets (PharMingen-BD). The colorimetric reaction was obtained after incubation with tetramethylbenzidine substrate reagent set. Doubling dilutions of recombinant mouse IL-1β or TNF-α ranging from 5 to 2000 pg/ml were used as standards. Optical density was measured at 405 nm with Dynatech MR5000 Reader (Long Island Scientific, East Setauccket, NY).
Cell treatments and reagents

MG, activated MG, CM, CM* or the indicated cytokines were applied to astrocyte cultures for 24 h. When mentioned, CM and astrocytes cultures were preincubated (2 h) with soluble TNF-α receptor (sTNF-αR1) and IL-1β receptor antagonist (IL-1ra), respectively, before incubation with proinflammatory cytokines or diluted CM* (24 h). IL-1β, TNF-α, IL-1ra, and TNF-αR1 were purchased from R&D Systems (Minneapolis, MN) and prepared as stock solutions in PBS and 1% BSA at 2 μg/ml, 10 μg/ml, 10 μg/ml, and 100 μg/ml, respectively. The Aβ25–35 and Aβ35–25 peptides were purchased from Bachem (King of Prussia, PA) and prepared in H2O at 1 mg/ml.

Statistical analysis

For each data group, results are expressed as means ± SEM and n refers to the number of independent experiments. For statistical analysis, each treatment was compared with its respective control, and significance was determined using a one-way ANOVA followed in case of significance by a Newman-Keuls post hoc test to compare the mean values of each group. Differences were considered significant at *P < 0.05; **P < 0.01; and ***P < 0.001.

RESULTS

Activated microglial cells inhibit gap junctional communication in astrocytes through the release of soluble factors

Interaction between activated MG and astrocytes was first studied in spontaneous mixed cultures. After 3 weeks, these cultures were composed of a confluent monolayer of GFAP-positive astrocytes with MG, identified by isolectin B4 staining, lying on the top or inserted between astrocytes (Fig. 1Aa, Ac). The percentage of MG vs. astrocytes was 38±20% (n=3). In these mixed cultures, the level of AGJC measured using the SL/DT technique was similar to that measured in astrocyte cultures (Fig. 1B). In contrast, after LPS treatment of mixed cultures (10 ng/ml, 24 h), the diffusion of LY was reduced by 65%, while similar treatment of astrocyte cultures had only a slight effect (5% inhibition). To reach a similar level of AGJC inhibition (69%) in astrocyte cultures, a higher concentration of LPS (10 μg/ml) was required. This observation indicates that in mixed cultures the effect of low LPS concentration is mediated by the activation of MG (Fig. 1B), although it cannot be ruled out that astrocytes get more sensitive to LPS in the presence of MG. Indeed, the effect of high doses of LPS on astrocyte cultures is unlikely due to the stimulation of few contaminating MG in astrocyte cultures (2±1%, n=3), since similar inhibition was observed either in cultures treated with L-leucine methyl ester (reported to eliminate residual MG) (12) or in secondary astrocyte cultures which, because of replating, were depleted in MG (not shown).

To better analyze the effect of MG on AGJC, either purified MG (10⁶ cells per dish) or 1 ml of CM were added to astrocyte cultures overlaid with 1 ml of culture medium. A significant decrease in AGJC level was observed 24 h after the plating of MG (up to 30%) whereas CM was without effect (Fig. 2A). In contrast, when activated by LPS, MG produced a strong inhibition (74%) of AGJC (Fig. 2A). The inhibitory effect of activated MG was likely due to the release of soluble factors because: 1) an inhibition was still observed when physical contact between the two cell types was prevented by culturing activated MG on inserts for 24 h and 2) the incubation
with CM* resulted in a strong inhibition (70%) of AGJC. In contrast, when these two protocols were performed using untreated MG, no significant change in AGJC was detected (Fig. 2A). Furthermore, a dose-dependent effect of successive dilutions of CM* samples led to the following reductions in AGJC: 61% for 1/4, 22% for 1/8, 19% for 1/16, 19% for 1/32, and 5% for dilutions ranging from 1/40 to 1/64 (n ranging from 4 to 6).

**Activated microglial cells down-regulate Cx43 expression in astrocytes**

Immunoblotting and immunocytochemical analysis were performed to determine whether decrease in GJC was related to changes in the pattern of Cx43 expression. Typically, in Western blots carried out with extracts of cultured astrocytes, three distinct bands were detected corresponding to the nonphosphorylated (NP) and the two phosphorylated (P1 and P2) forms described previously for Cx43 in astrocytes (10). Treatment carried out with LPS (10 ng/ml) had no effect on cultures of astrocytes alone, but when activated MG were added for 24 h on astrocyte cultures, the amount of Cx43 was significantly reduced without change in the proportion of the three Cx43 forms (Fig. 2B). Evidence that Cx43 is solely expressed in astrocytes was demonstrated by the lack of Cx43 detection in MG or activated MG alone treated with LPS for 6 or 24 h (Fig. 2B). Similarly, Cx43 was not detected in MG and LPS-activated MG with either a polyclonal anti-Cx43 antibody or with the mouse anti-Cx43 mAb routinely used. In agreement with dye-coupling data, CM application had no effect on Cx43 expression, whereas CM* strongly reduced the amount of Cx43. In addition, MG cultured on insert did not affect Cx43 expression, while a marked reduction was obtained with activated MG (Fig. 2C). Finally, the expression of Cx43 investigated by immunofluorescence, was also decreased in mixed cultures after LPS treatment (Fig. 1Ab and 1Ad, Table 1). Again, no Cx43 immunofluorescence staining was detected in MG from mixed cultures treated or not with LPS.

**Coapplication of IL-1β and TNF-α inhibits intercellular communication and Cx43 expression in astrocytes**

Activated MG are known to be the source of a number of immunoregulatory mediators (13), and there is already evidence that a recombinant proinflammatory cytokine affects astrocyte gap junctions (14–16). Accordingly, the effect of four compounds potentially released by activated MG was tested on AGJC. When applied individually, IL-6, INF-γ, and TNF-α (10 ng/ml, 24 h) did not result in a significant change in AGJC (even at concentration up to 100 ng/ml, not shown), while IL-1β produced a weak inhibition (Fig. 3A). Alternatively, coapplication of these compounds was performed and among all possible combinations tested, only the coapplication of TNF-α and IL-1β (10 ng/ml, 24 h) strongly reduced AGJC with more than an additive effect. These observations were completed by an immunoblotting analysis that demonstrated a strong down-regulation of Cx43 expression in astrocytes treated with TNF-α and IL-1β, indicating that the inhibition of dye-coupling likely resulted from a decrease in the number of gap junction channels (Fig. 5D). This cytokine-induced down-regulation of Cx43 expression was confirmed by immunofluorescence analysis (Table 1).
TNF-α and IL-1β are involved in the inhibitory effect of the conditioned medium harvested from activated microglial cells

To determine whether TNF-α and IL-1β are released by activated MG, their respective levels were assessed by ELISA assays performed on CM and CM*. Although no production could be detected in control condition, 2.7 ± 0.7 ng/ml of IL-1β and 22.7 ± 6.8 ng/ml of TNF-α were measured (n=9) in CM*. When applied on astrocytes at the respective concentrations of 2 ng/ml for IL-1β and 20 ng/ml for TNF-α, these cytokines also generated a significant reduction in AGJC (62% inhibition, n=3).

To evaluate the involvement of these cytokines in the CM*-induced inhibition of AGJC, specific blockers of each cytokine activity, IL-1ra and sTNF-αR1 (the recombinant receptor antagonist for IL-1β and the soluble receptor for TNF-α, respectively), were used to antagonize their effect. The treatment of astrocytes with CM* used at 1/4 dilution (a dilution that has a marked effect on AGJC) in presence of either IL-1ra or sTNF-αR1 (100 ng/ml) resulted in a partial reduction of the inhibition of AGJC (27% and 23% inhibition, respectively, n=4). Moreover, in the presence of both agents, the inhibition induced by 1/4 diluted CM* was abolished (15% inhibition, n=4). These observations demonstrate that these two proinflammatory cytokines contribute to the CM*-induced inhibitory effect on AGJC. Indeed, when the inhibition of AGJC was provoked by the coapplication of IL-1β and TNF-α, similar effects of IL-1ra or sTNF-αR1 were obtained: the resulting inhibition of AGJC was 43% in presence of IL-1ra, 31% with sTNF-αR1 and 9% when applied together (n=3).

β-amyloid potentiates the inhibitory effect of conditioned medium from activated microglia on astrocyte gap junctions: role of IL-1β and TNF-α

In AD, an inflammatory response characterized by MG activation and reactive astrocytes typically occurs at sites of Aβ deposits. Furthermore, Aβ is known to evoke the release of IL-1β and TNF-α from MG, although in lower amounts than LPS (17). In MG cultures, treatment with Aβ25-35 (20 μM, 24 h), the active fragment of Aβ, evoked a weak release of IL-1β and TNF-α from MG (109.3 and 46.5 pg/ml, respectively; n=3). Because Aβ is localized at sites where proinflammatory cytokines are potentially released, coapplication (24 h) of Aβ25-35 with either IL-1β or TNF-α or both were tested on AGJC. Although IL-1β or TNF-α had a slight or no significant inhibitory effect on their own (16% and 7%, respectively, n=5), the coapplication of each cytokine with 20 μM Aβ25-35 resulted in a significant inhibition of AGJC (71% and 73%, respectively; n=5) (Fig. 4A). Moreover, the inhibitory effect of these cytokines applied together was enhanced in the presence of Aβ25-35 (Fig. 4A). Interestingly, while the effect of CM* diluted to 1/40 to 1/64 was not significant, its coapplication with Aβ25-35 resulted in a marked AGJC inhibition (59%, n=7). In contrast, this inhibition was not observed when Aβ35-25, the nonactive reverse sequence of the peptide, was substituted to Aβ25-35 (4%, n=3).

Such inhibitory effect produced by the coapplication of diluted CM* and Aβ25-35 was correlated with a decrease in the level of Cx43 expression analyzed by immunoblotting and immunofluorescence. Indeed, Western blot analysis demonstrated that a drastic down-regulation in Cx43 expression occurs when either IL-1β or TNF-α or both were coapplied with Aβ25-35 (Fig. 5A, Table 1). Moreover, the quantification of the distribution of immunoreactive dots indicated that
the level of Cx43 expression in astrocytes was weakly affected in the presence of diluted CM* or Aβ25−35 alone, while it was significantly decreased after their coapplication (Fig. 5B–E, Table 1).

Finally, the inhibition induced by the coapplication of diluted CM* and Aβ25−35 was significantly prevented in the presence of IL-1ra or sTNF-αR1 used at concentrations that were shown to block the inhibitory effect of IL-1β and TNF-α coapplication. In this set of experiments AGJC was reduced by 59% by the coapplication of diluted CM* and Aβ25−35, whereas it only reached 29% in the presence of IL-1ra or sTNF-αR1 (Fig. 4B).

**DISCUSSION**

In vitro, as well as in brain slice preparations, the high level of AGJC has led us to propose that astrocytes are organized as a communicating network (4), the extent of which is controlled by a number of endogenous factors (5). Interestingly, neurons were shown to increase Cx43 expression and AGJC (10), while MG exert the opposite effect (9). In addition, it was suggested that this inhibitory effect depended on the inflammatory status of MG estimated by morphological analysis (18, 19). In the present work, the demonstration that MG activation is indeed critical was achieved either by LPS treatment of mixed cultures or by adding LPS-activated MG on astrocyte cultures or conditioned medium harvested from LPS-activated microglia. In addition, we identified the mechanism by which activated MG control astrocyte gap junctions and Cx43 expression. Activated MG are 4 times more efficient than untreated MG in down-regulating Cx43 expression and AGJC, and these effects are mediated by soluble factors released by activated MG. The main factors involved in this inhibition are two proinflammatory cytokines, IL-1β, and TNF-α. Compared with previous studies (14–16, 19) that were performed using recombinant cytokines, we were able to demonstrate that native cytokines released from activated MG were also efficient. Nevertheless, the contribution of other secreted factors cannot be ruled out, as, for instance, nitric oxide and chemokines, which are also produced by MG or astrocytes in response to stimulation by bacterial agents or by cytokines such as TNF-α and IL-1β (20, 21). The finding that two identified proinflammatory cytokines down-regulate Cxs expression and function in astrocytes completes previous observations demonstrating that these molecules affect several glial functions (22, 23). This includes GJC in central (8) and peripheral (24) glial cells, a property that is shared with a number of other systems (25) defining gap junctions as important targets in inflammatory processes.

The combination of several culture model systems and the use of CM have allowed us to demonstrate that Aβ25−35 potentiates the inhibitory effect of MG products on astrocyte gap junctions. As the inhibition of AGJC and Cx43 expression is observed when diluted CM* and Aβ are coapplied on astrocytes cultures, that is, in the absence of MG, this potentiation may directly operate at the level of astrocytes. These observations are of particular interest because a critical step in AD is apparently reached when pathological glial activation is not restricted to MG but includes astrocytes (26). However, it is not excluded that other pathways lead to a similar block of AGJC. Because IL-1β and TNF-α were shown to induce the release of TNF-α from astrocytes (23), such secondary production of this cytokine could therefore amplify the effect of activated MG on AGJC. Alternatively, Aβ is also known to induce the release of proinflammatory cytokines from MG (this study; 17), although in much lower amounts than under LPS stimulation. In this case, AGJC inhibition could also result from the effect of Aβ on MG, leading to the production of a small amount of proinflammatory agents, sufficient to
potentiate its effect on AGJC. Indeed, as already stated, Aβ at senile plaques can amplify glial reaction by a coexisting inflammatory stimulus (27). Whatever the cellular mechanism leading to the potentiation of IL-1β or TNF-α by Aβ, the present study indicates that Aβ exerts its potentiation by modifying the threshold of astrocyte response to proinflammatory cytokines, as already reported for the induction of nitric-oxide synthase in astrocytes (28). Such diversity of pathways indicates that interactive mechanisms between astrocytes and MG are likely complex and may depend upon the respective location of the two glial partners and the time at which the process of reactive gliosis is considered. Indeed, timing is likely to be an important parameter as it was shown that Cx43 expression is increased in astrocytes at the proximity of amyloid plaques in brain from AD patients (29). These features indicate that status of astrocyte gap junctions may vary according to the progression of the disease.

It has been reported that AGJC propagates intercellular signals that lead to phenotypic transformation and cell injury in astrocytes (30, 31). Furthermore, gap junction channels remain open in dying astrocytes (32), suggesting that AGJC could contribute to the propagation of death signals at least between astrocytes. However, up to now, the consequence of such propagation has not been investigated in terms of neuronal survival. In contrast, there is increasing evidence arguing that AGJC could rather play a neuroprotective role. Indeed, it has been demonstrated that noncommunicating astrocytes are less neuroprotective than astrocytes with functional gap junctions, for instance the inhibition of AGJC enhances the neurotoxicity of oxidative agents (33) and glutamate (34). Moreover, after a stroke lesion, the volume of brain infarct is larger (35), and an increased neuronal apoptosis is observed several days after focal ischemia in heterozygote Cx43 knockout mice (36). Thus, AGJC could contribute to the neuroprotective role of astrocytes after CNS damages. Consequently, the inhibition induced by the coapplication of proinflammatory agents and Aβ could account for the proposed glial contribution to neurodegenerative processes in AD (37). However, the alteration in neuroprotection due to astrocyte dysfunctions could also result from changes in other astrocytic properties that participate to brain homeostasis and are known to be affected by proinflammatory cytokines and Aβ. For instance, glutamate uptake is impaired by IL-1β and TNF-α (38) and by Aβ (39), intercellular calcium signaling through gap junctions is reduced by IL-1β, while the ATP-mediated component is increased (40) by IL-1β and by Aβ (41), and glucose utilization is stimulated by IL-1β and TNF-α (42), while AD patients show an hypometabolism (43). Even in these cases, it is noteworthy that AGJC could play a role since it has been proposed to contribute to the control of glutamate homeostasis, the propagation of intercellular calcium waves and the supply of metabolic substrates (44–46).

Finally, although the present data were obtained from in vitro experiments, this glial interaction resulting in the inhibition of AGJC can be considered in the more general context of the progression of neurodegenerative diseases. Indeed, this inhibitory effect may play a role in an amplification phenomenon leading to the extension of neuronal damages. As activated MG inhibit AGJC and neuronal death has been reported to down-regulate Cx43 expression and AGJC (10), both effects may result in a decrease in the neuroprotective role of astrocytes. Then as neuronal death is associated with a glial reaction, a repetition of the above steps could take place by establishing a "vicious circle". In this context, it will be critical to determine whether the inhibition of astrocyte gap junctions reported here affects neuronal survival and disrupts neuron-glial interaction, a situation that is an important contributing factor to neurodegeneration. Thus, alternative strategies to prevent, or at least reduce, the progression of neurodegenerative diseases
should also target glial cells with the objective to preclude processes that result in the inhibition of gap junction-mediated communication in astrocytes.

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REFERENCES


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Table 1

Effect of pro-inflammatory treatments, conditioned medium harvested from LPS-activated microglia and Aβ25-35 on Cx43 expression in astrocyte cultures

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Immunofluorescence analysis was performed by counting the number of immunoreactive dots per area and by Western blot analysis using semiquantitative densitometry analysis. All data are expressed as the percentage of the values obtained in untreated astrocyte controls. *n* = number of independent experiments.
Figure 1. LPS addition in mixed culture of astrocytes and microglia inhibits Cx43 expression and gap junctional communication in astrocytes. *Aa–Ad* Confocal images showing in red the expression of GFAP (*Aa, Ac*) and Cx43 (*Ab, Ad*) in astrocytes and, in green MG identified by Isolectine B4 staining, in the absence (*Aa, Ab*) and presence (*Ac, Ad*) of LPS (10 ng/ml, 24 h). Scale bar: 20 µm. *B* AGJC was evaluated in primary cultures of astrocytes and in mixed cultures of MG and astrocytes using the SL/DT. The inhibitory effect of LPS treatment was strong in mixed cultures and weak in astrocytes cultures where higher LPS concentration was required to reach a similar level of inhibition.
Figure 2. LPS-activated microglial cells inhibit gap junctional communication and Cx43 expression in astrocytes. AGJC was evaluated by SL/DT in primary cultures of astrocytes where nontreated (+MG) or LPS-activated MG (+MG*) were seeded overnight. A) Inhibitory effect of MG on AGJC requires physical contact between the two cell types: the direct addition of MG reduced the level of AGJC while MG addition into cell inserts (+MG insert) did not have a significant effect. In contrast, seeding MG* to astrocyte culture (+MG*) or into cell insert (+MG* insert) induced a strong inhibition. Finally, the addition of conditioned media (1/2 dilution) harvested from either MG (CM) or activated MG (CM*) showed that only the latter was efficient in reducing AGJC. Data were obtained from 3–14 independent experiments. B, C) Western blot analysis of Cx43 expression in protein extracts from various cell treatment. B) Down-regulation of Cx43 in astrocytes when MG were stimulated with LPS at 10 ng/ml (+MG*) or 1 µg/ml (+MG**) before seeding on astrocyte cultures. In contrast, no Cx43 was detected in purified MG exposed to similar concentrations of LPS (MG* and MG**). C) Compared with the untreated astrocyte cultures, the level of Cx43 expression was weakly altered by the direct addition of MG (+MG) and strongly reduced by that of LPS-activated MG (+MG*) even seeded into cell insert (MG* insert) or by conditioned medium (1/2 dilution) harvested from activated MG (CM*). In this experiment, membranes were also hybridized with anti-α-tubulin antibody to visualize the amount of protein loaded for each sample.
Figure 3. Effect of proinflammatory cytokines on AGJC and Cx43 expression in astrocytes. Interleukin-1β (IL1), tumor necrosis factor-α (TNF), interleukin-6 (IL6), and interferon γ (IFN) were applied on cultured astrocytes, alone or in combination. From all possible combinations, only the coapplication of IL-1β + TNF-α resulted in a strong inhibition of AGJC, while only IL-1β had a slight but significant effect. Data were obtained from 3–5 independent experiments.
Figure 4. Amyloid β_{25-35} potentiates the effect of proinflammatory cytokines AGJC. A) The presence of Aβ_{25-35}, which has no effect by itself on AGJC, induced an inhibition when coapplied with either IL-1β or TNF-α enhanced the blocking effect of IL-1β and TNF-α. B) A similar potentiation was observed when Aβ_{25-35} was coapplied with diluted CM* (Dil.CM*). The dilution of the CM* varied from 1/40 to 1/64 depending on the efficiency of the selected sample and was chosen at the threshold dilution found to have no significant effect on AGJC. This potentiation was blocked in the presence of IL-1ra and sTNFαR1 (Aβ+Dil.CM*+2antag). Data were obtained from 3–18 independent experiments.
Figure 5. Potentiation of proinflammatory cytokines or conditioned medium collected from activated MG with amyloid β_{25-35} on Cx43 expression in astrocytes. A) Western blot analysis of Cx43 and α-tubulin expression was performed in primary cultures of astrocytes treated with different combinations of IL-1β, TNF-α, and Aβ. As for AGJC, while cytokines applied alone had a mild effect, a strong inhibition was evoked by the coapplication of IL-1β and TNF-α with Aβ_{25-35}. B–E) Confocal images of Cx43 immunoassaying of GFAP-labeled astrocyte cultures in control conditions (B) or after exposure to Aβ_{25-35} (C), diluted CM* (D) and both (E). Scale bar: 20 µm. These results are representative of at least 3 independent experiments.