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Microglial Morphology and Its Transformation After Challenge by Extracellular ATP In Vitro

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The novel morphological characteristics of N9 microglial cells and primary cultured rat microglial cells were examined using the bitotin-IB4 and streptavidin-FITC system. Numerous fine, long processes of both microglial cell preparations formed a network, beginning after 30 min in culture. Dye coupling studies did not show communication between neighbouring cells via the processes in normal conditions. The network of microglial cell processes was well formed into a 'resting state' by 16–24 hr after re-plating. After being challenged by 3 mM ATP the microglial cells were activated, became amoeboid-like cells within 2 hr and finally floated in the culture medium. The complicated network of processes did not retract to the microglial cell body. Flow cytometry analysis showed that the majority of these floating cells were alive and could recover to the resting state after ATP was removed from the culture medium. © 2005 Wiley-Liss, Inc.

Key words: flow cytometry; P2 receptor; culture; ramified cells; suramin

Microglia are distributed throughout the central nervous system (CNS) as a network of resting immunocompetent cells derived from the monocyte/macrophage lineage, comprising 5–15% of the total cells in the brain (Lawson et al., 1990). In the healthy brain, microglia have a typical ramified morphology that does not occur in any population of macrophages in other tissues. This ramified phenotype consists of a small cell body, long slender processes with secondary branching and lamellipodia. By exploiting a silver carbonate stain, del Rio-Hortega (1932) was the first to identify microglia as a specific class of argentophilic brain cells with two or more branched processes and spines. Del Rio-Hortega further described microglia during embryonic development as amoeboid cells showing such macrophagic properties as migratory capacity and phagocytosis. During CNS injury or inflammation, the ramified microglia processes retract, become more amoeboid-like, and transform into a third form, the reactive microglia (del Rio-Hortega and Penfield, 1927; Aloisi, 2001). The mechanism underlying the morphological transformation from ramified microglia into amoeboid-like microglia is not known.

In the present study we have used histochemical staining with isolectin B4-FITC to study a murine microglia cell line (N9) and primary cultured rat microglial cells. We have found that the microglial cells, mimicking at least in part resting microglia, were a suitable cell model to study the transformation into reactive microglia. Microglial cells have been found to expresses P2X purinoceptors (Bianco et al., 2005a; Xiang and Burnstock, 2005) and extracellular ATP can activate microglia, causing morphological changes with membrane ruffling (Inoue, 2002). We used ATP as an activator to study the morphological transformation from resting to activated states.

MATERIALS AND METHODS

Cell Culture

The N9 murine microglial cell line was a kind gift from Dr. P. Ricciardi-Castagnoli (Universita Degli Studi di Milano-Bicocca, Milan, Italy). These cells have been extensively used as representatives of mouse microglial cells (Ferrari et al., 1996, 1997; Cui et al., 2002; Zhang et al., 2003). The cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μm 2-mercaptoethanol (2-ME) cultured at 5% CO_2 and 37°C. The cells were fixed with 4% paraformaldehyde prepared

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in phosphate buffered saline (PBS), for 30 min and then used for histochemical staining.

Rat microglial cells were obtained from primary cell cultures of cerebral hemispheres of neonatal rats (P1–P3 rats) as described previously (Nakajima et al., 1989, 1992). Primary mixed cells were cultured in DMEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% foetal bovine serum (FBS). After 2–3 weeks, microglial cells were prepared as floating cell suspensions by shaking growth flasks on an orbital shaker (100 r.p.m., 30 min). For the histochemistry staining study, aliquots of the cell suspension ($1–2 \times 10^4$ cells) were transferred to wells of an 8-well chamber slide (Nunc, Naperville, IL) and allowed to adhere at 37°C. After 15 min, unattached cells were removed by rinsing twice with serum-free DMEM. After the final rinse, 0.3 ml mixed medium was added to each well and the slides kept at 5% CO_2 at 37°C. The purity of microglia was almost 100%. The mixed medium was composed of half volume of DMEM with 10% heat-inactivated FBS, 2 mM of glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and half volume of glial-conditioned medium prepared by placing DMEM with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS into a flask of confluent primary cultured brain cortical mixed cells of newborn rats for 72 hr. The cells were fixed with 4% paraformaldehyde prepared in PBS, for 30 min and then used for histochemical staining.

ATP Stimulating Experiments

For the morphological study, N9 microglial cells and primary cultured microglial cells were cultured in 8-well chambered slides at the cell density of $1–2 \times 10^4$/well for 16 hr after re-plating. Two hours before stimulation with ATP, the culture medium was replaced with serum-free DMEM. ATP (Sigma Chemical Co., Poole, UK) was used at a concentration of 3 mM and the P2 receptor antagonist suramin (Sigma) was used at a concentration of 100 μM. 0 min (as control), 30 min, 1 hr, 1.5 hr, and 2 hr after stimulation with ATP the cells were fixed with 4% paraformaldehyde prepared in PBS for 30 min at room temperature. In a separate group, the cells were preincubated for 30 min with suramin, and then challenged with ATP. Cells were fixed with 4% paraformaldehyde prepared in PBS for 30 min at room temperature for 30 min, 1 hr, and 2 hr after stimulation with ATP.

For flow cytometry analysis, N9 microglial cells were cultured in 6-well plates at a cell density of 10^6 cells/well for 16 hr. Two hours before stimulation, the culture medium was replaced with serum-free medium. The detached cells were collected and washed two times with DMEM before an Annexin V Binding Assay 30 min, 1 hr, and 2 hr after stimulation with 3 mM ATP. In a control group, the cells were not challenged with ATP, just rinsed in serum-free DMEM. The cells were treated with 0.25% Trypsin-EDTA (Sigma) for 15 min at 37°C, washed two times with DMEM before the Annexin V Binding Assay.

Histochemistry

Primary cultured microglia cells and N9 microglial cells were incubated with biotinylated isolectin B4 (Sigma) diluted 1:200 in antiserum dilution solution, overnight at 4°C. Subsequently the specimens were incubated with FITC-conjugated (Amersham Plc, Little Chalfont, UK) diluted 1:200 or peroxidase-conjugated streptavidin (Sigma) diluted 1:1,000 in antiserum dilution solution for 2 hr at room temperature. For the fluorescence, the specimens were mounted in buffered glycerol (Sigma). A nickel-intensified diaminobenzidine (DAB) reaction in a solution containing 0.05% 3,3′-DAB, 0.04% nickel ammonium sulphate, 0.2% glucose, 0.004% ammonium nitrate, and 1.2 u/ml glucose oxidase in 0.01 M PBS was used to visualize peroxidase reactivity. All the incubations and reactions were separated by 3×10 min washes in PBS.

Single Microglial Cell Injected With Lucifer Yellow

A culture dish was placed on the stage of an inverted microscope (Diaphot, Nikon) and cells were visualised under phase contrast at 600× magnification. The culture dish was perfused at a rate of 0.5 ml min^{-1} with extracellular solution containing (mM): NaCl, 154, KCl 4.7; HEPES, 10; D-glucose, 5.6; $CaCl_2$, 2.5; $MgCl_2$ 1.2 and adjusted to pH 7.4 with NaOH. Whole cell patch clamp recordings were made using pipettes fabricated from thin wall borosilicate glass capillaries (Clark Electromedical, GC 150TF) that had a resistance of 2–4 MΩ when filled with internal solution containing (mM): KCl, 120; HEPES, 10; tripotassium citrate, 10 and EGTA 0.1; the pH was adjusted to 7.2 using KOH, to which had been added 1% Lucifer Yellow (lithium salt; Sigma). Membrane currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Union City, CA), and the access resistance continually monitored by imposing 10 mV hyperpolarizing voltage commands. After satisfactory whole cell recording lasting for 2–5 min, the electrode was carefully withdrawn from the cell. After a further period of 10–30 min, the cells were fixed with 4% paraformaldehyde PBS, and viewed with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany).

Photomicroscopy

Images were taken with the Leica DC 200 digital camera (Leica) attached to a Zeiss Axioplan microscope (Zeiss). Images were imported into a graphics package (Adobe Photoshop 5.0, San Jose, CA). The lengths of processes and cell body sizes under different conditions were measured using a FR 988 system (a computer image analysis system designed by Shanghai FURI Science and Technology Co., Ltd, Shanghai, P.R. China).

Flow Cytometry

The Annexin V Binding Assay was used to detect live, necrotic and apoptotic cells; the cells were examined after treatment with 3 mM ATP. The cells (5×10^5 cells/treatment) were used to determine the translocation of phosphatidylserine to the outer surface of the plasma membrane during apoptosis using the human phospholipid binding protein, annexin V, conjugated with fluorescein (Roche-Boehringer-Mannheim Corp., Indianapolis, IN) by flow cytometry as described by the manufacturer. Live, apoptotic, and necrotic cells were analyzed by quadrant statistics on the propidium iodide-negative, fluorescein isothiocyanate-negative cells; the

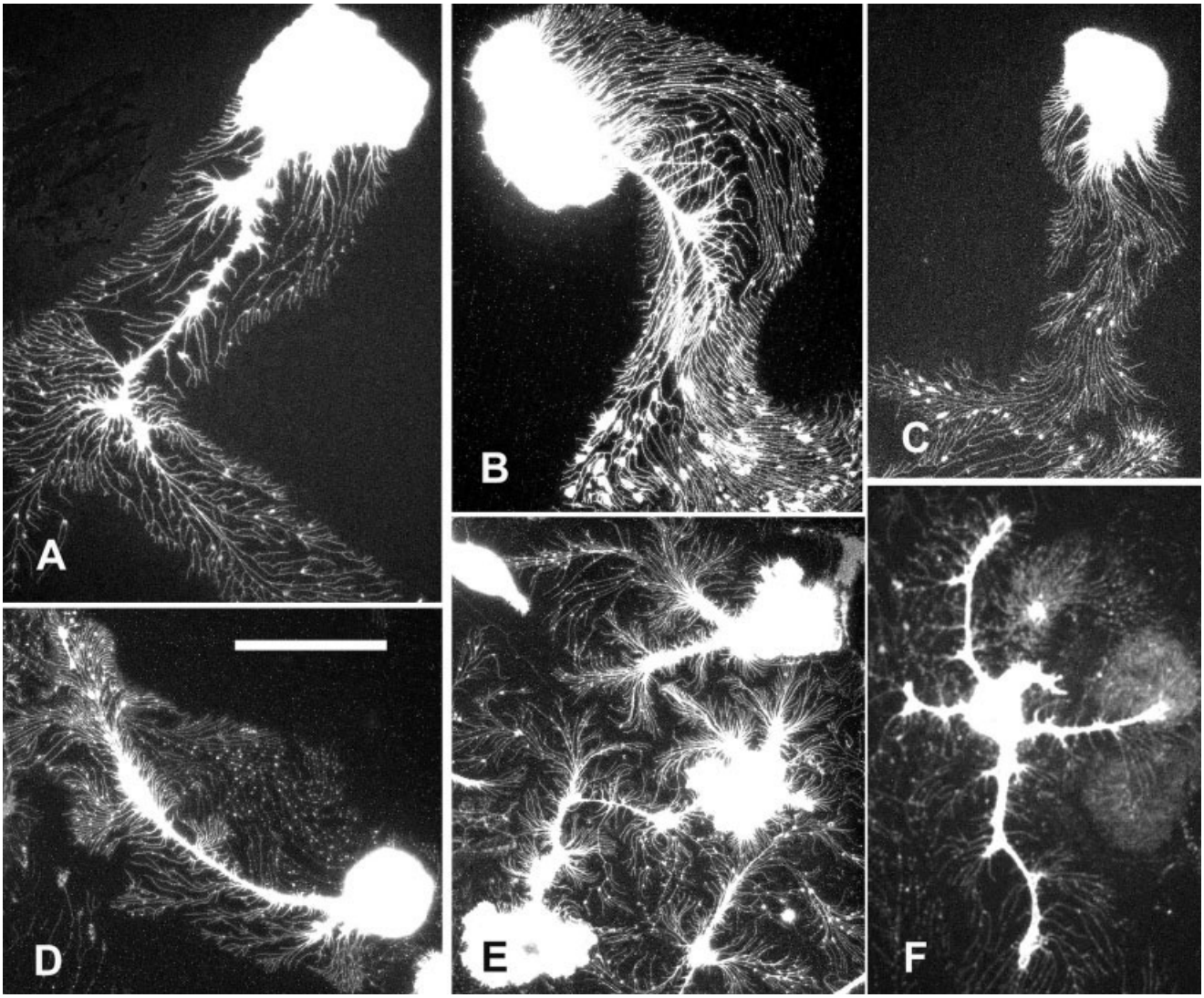

Fig. 1. The morphological characteristics of primary cultured rat microglial cells and N9 microglial cells shown by Biotin-IB4 and Streptavidin-FITC staining. **A:** Primary culture of a rat microglial cell with a long primary process, from which many spine-like processes originate, and many long fine processes branch out from the primary process, spine-like processes and the cell body. The diameter of the fine processes are approximately 0.5 μm. The fine processes may arborize further, 1–5 times 24 hr after re-plating. **B:** N9 microglial cell, of which the morphological characteristics are similar to (A), but some areas of the fine processes had expanded further 24 hr after re-plating. **C:** Primary cultured microglial cell. This microglial cell had no primary processes, but had many fine processes (24 hr after re-plating). **D:** N9 microglial cell with a long primary process and numerous fine processes (24 hr after re-plating). **E:** Primary cultured rat microglial cells. A network of interrelated microglial cell processes from three cell bodies 48 hr after re-plating. **F:** N9 microglial cell with five primary processes and numerous fine processes 48 hr after re-plating. Scale bar = 50 μm.

propidium iodide-negative, fluorescein isothiocyanate-positive cells, and propidium iodide-positive cells, respectively.

RESULTS

The morphology characteristics of N9 microglial cells shown by the bitotin-IB4 and streptavidin-FITC system in the present study are novel. Typically the N9 microglia cells had polygonal or oval cell bodies with 0–5 primary processes. Numerous fine and long secondary processes were found to branch from the cell body and primary process. These processes bifurcate further up to 6 times. There are knot-like structures in some cross points of branches. These knot-like structures were brighter than other areas of the processes. The fine processes usually sprouted from one side of the cell body. The diameter of these processes was approximately 0.2∼0.5 μm and the length from the primary processes was up to 150 μm. The length of these processes was longer in those microglial cells cultured for 24–48 hr. A network of the microglial cell processes arising from the cell body was clearly

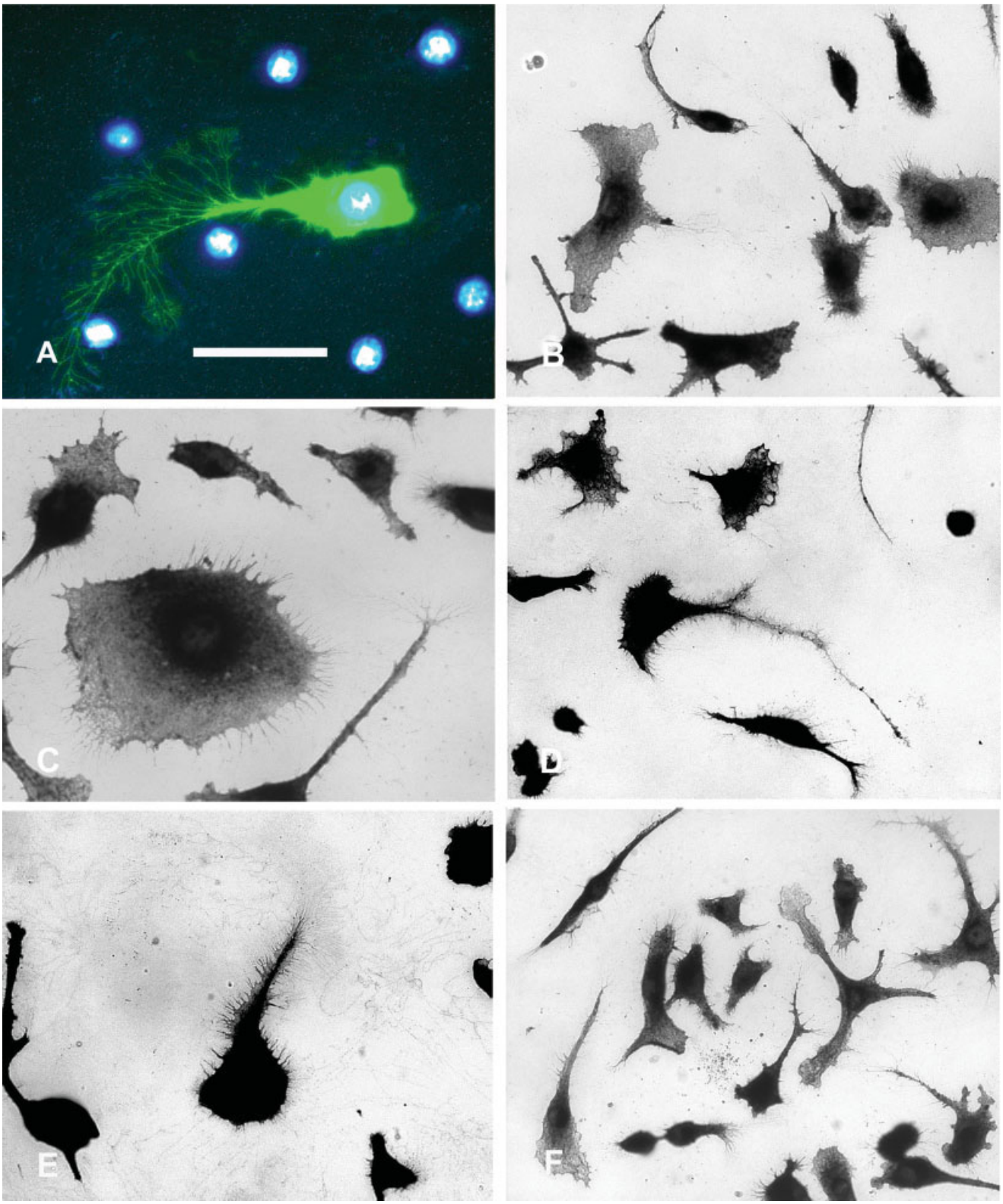

Fig. 2. Single cell injection of Lucifer Yellow and the morphological characteristics of the primary cultured rat microglial cells and N9 microglial cells shown by Biotin-IB4 and Streptavidin-FITC staining. **A:** N9 microglial cells labeled with Lucifer Yellow and counterstained with DAPI. Note that there are no neighbouring cells or cell processes labeled by Lucifer Yellow, the small round structures are nuclei labeled by DAPI. **B–D:** N9 microglial cells. **E,F:** Primary cultured rat microglial cells labeled with Biotin-IB4 and Streptavidin-FITC. In (B–F) the spine-like structures and fine processes were also demonstrated, but not as clearly as those in Figure 1 labeled by Biotin-IB4 and Streptavidin-FITC system. Scale bar = 50 μm.

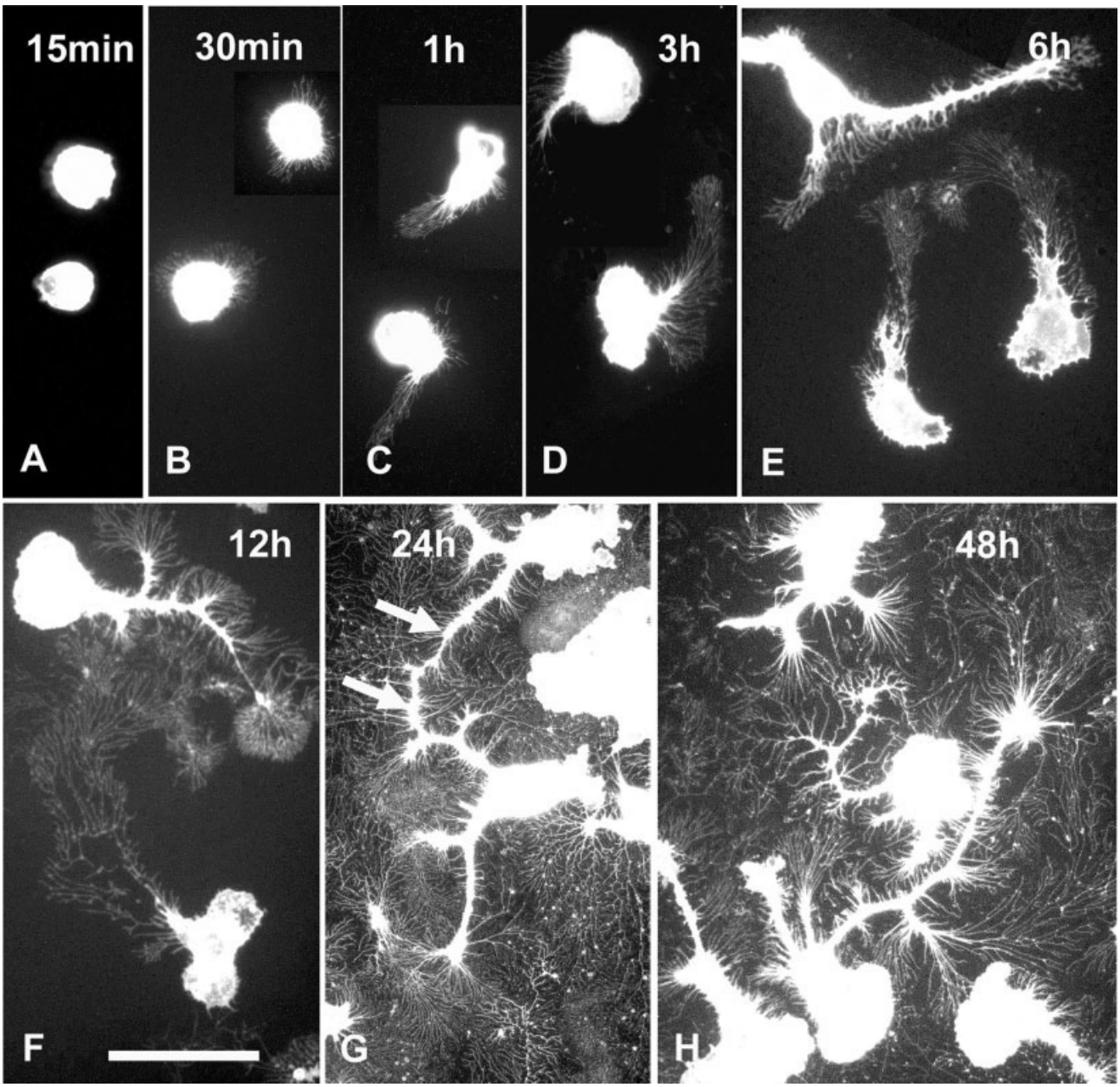

Fig. 3. The morphological characteristics of N9 microglial cells at different time from 15 min to 48 hr after re-plating. **A:** N9 microglial cells 15 min after re-plating. Note that there are no processes on the round cell body at this time. **B:** N9 microglial cells 30 min after re-plating. Note that there are numerous short fine processes from the round cell body at this time. **C:** N9 microglial cells at 1 hr after re-plating. Note that microglial cell body begins to change from a round or oval shape to an irregular shape. The fine processes grow longer and are polarized on one side of the cell. **D:** N9 microglial cells 3 hr after re-plating. Note that a primary process has appeared, the cell bodies are flattened and the fine processes have continued to grow longer. **E:** N9 microglial cells 6 hr after re-plating. Note that the primary processes are longer, there are many spine-like structures on the long primary process, the cell bodies are further flattened and the fine processes have continued to grow longer. **F:** N9 microglial cells 12 hr after re-plating, Note that the fine processes have grown much longer. **G:** N9 microglial cells 24 hr after re-plating. Note that the fine processes form a network, the linked primary processes from the neighbouring two microglial cells are shown by arrows. **H:** N9 microglial cells 48 hr after re-plating. Note that the fine processes, primary process and cell bodies are similar to those 24 hr after re-plating. Scale bar = 50 μm.

TABLE I. Length of Fine Processes of Microglial Cells at Different Times After Re-plating[†]

	15 min	30 min	1 hr	3 hr	6 hr	12 hr	24 hr	48 hr
N9	0	6 ± 3★★	22 ± 5★★	34 ± 7★	75 ± 10★★	95 ± 13★	131 ± 4★★	134 ± 11
P	0	5 ± 2★★	18 ± 6★★	27 ± 8★	65 ± 11★★	87 ± 9★	125 ± 10★	130 ± 8

[†]The longest process was measured from a randomly selected cell and 150 cells were selected from one experiment. Four independent experiments were carried out. The data represent the mean ± SEM (μm). P, primary culture; N9, mouse microglial cell line.
★$P < 0.05$, ★★$P < 0.01$ with respect to the former time group, analysed by Student's *t*-test.

demonstrated by the biotin-IB4 and FITC-streptavidin system. Some of these fine processes seemed to be connected with processes originating from other microglial cells or with other cell bodies (Fig. 1). To determine whether those closely aligned cell processes were communicating directly, Lucifer Yellow was injected into 10 microglial cell bodies, but no neighbouring microglial cells showed staining (Fig. 2A). The fine processes did not show as clearly as some stained with biotin-IB4 and streptavidin-peroxidase as seen in Figure 1 (Fig. 2B–F).

To determine when the fine processes first appear on the microglial cells after re-plating, the biotin-IB4 and streptavidin-FITC system was used to study the morphology of primary cultured microglial cells and N9 microglial cells after re-plating for 15 min, 30 min, 1 hr, 3 hr, 6 hr, 12 hr, 24 hr, and 48 hr. For most microglial cells their processes (although short) appeared at 30 min. At 1 hr all the microglial cells had processes and the processes were longer than those at 30 min. The processes grew longer with time (Fig. 3). Table I shows the average length of these processes at different times.

The bitotin-IB4 and streptavidin-FITC system was also used to study the microglial morphology and the process of morphological transformation. Thirty minutes after stimulation with ATP (3 mM), many new processes appeared radially around most of the N9 microglial cells and small bulbs formed in the ends of these new processes as compared to those in control group (Fig. 4A,B). It is possible that these bulbs were retracted processes. The cell bodies became round or oval. One hour after stimulation, most of the cell bodies were round or oval, and much smaller than those in control group. The new processes became shorter and the retracted bulbs were bigger than those in the 30-min group (Fig. 4C). One and a half hours after stimulation, almost all of the cell bodies were round and the new processes had disappeared, although most of the fine processes had not retracted to the cell bodies (Fig. 4D). Two hours after stimulation most of the microglial cell bodies had became detached, but the fine processes remained attached (Fig. 4F). In the group preincubated with suramin, the morphology of the N9 microglial cells did not change significantly (Fig. 4E). The lengths of the processes and the new processes at different times after stimulation with ATP, suramin or suramin plus ATP are summarized in Tables II–IV. The average size of the cell bodies (measured by maxim axes) decreased rapidly during the ATP stimulation (Table V). During ATP stimulation, the microglial cell bodies detached gradually and floated in the medium. Two hours after ATP stimulation most of the microglial cell bodies were detached. To determine whether these detached microglial cells were alive or dead, they were collected, labeled by the Annexin V-FLUOS Staining Kit and analyzed by flow cytometry (Fig. 5, Table VI). The results show that the majority of the detached cells were alive. These detached microglial cells were also re-plated and re-cultured for studying the morphological recovery after washing two times with DMEM. Thirty minutes after re-plating, the cells began to attach and about half of them were attached after 1 hr and the cells still floating in the medium were dead. The dead cells were removed by changing the medium. The cells that had attached were still able to ramify as previously (Fig. 6).

Fig. 4. The morphological changes of N9 microglial cells after stimulation with ATP (3 mM). **A:** Morphological characteristics of N9 microglial cells in a control group. **B:** Morphological changes of N9 microglial cells 30 min after being stimulated with ATP. Note that many of the new processes radiated from most of the microglial cells. At the ends of these new processes small bulbs have formed, these bulbs may be the retraction processes. **Inset b** (marked by dashed white lines) is a high magnification of the area that is indicated by a white arrow. **C:** N9 microglial cells 1 hr after stimulation with ATP. Note the round or oval cell bodies, which are smaller than those in control group shown in (A). The new processes are shorter and the retracted bulbs are bigger than those in the experimental group after 30 min exposure to ATP (B). **Inset c** (marked by dashed white lines) is a high magnification of the area that is indicated by a white arrow. **D:** N9 microglial cells 1.5 hr after stimulation with ATP. Note that most of the new processes have disappeared, although many of the fine processes are still present. **E:** N9 microglial cells 2 hr after preincubation with suramin (100 μM) followed by stimulation with ATP. Note that no significant changes of the morphology of microglial cells were observed. **F:** Remains after N9 microglial cells detached 2 hr after stimulation with ATP. Note that the fine processes did not detach at the same time as the cell bodies. Stars show the positions from which the cell bodies detached. Scale bar = 50 μm.

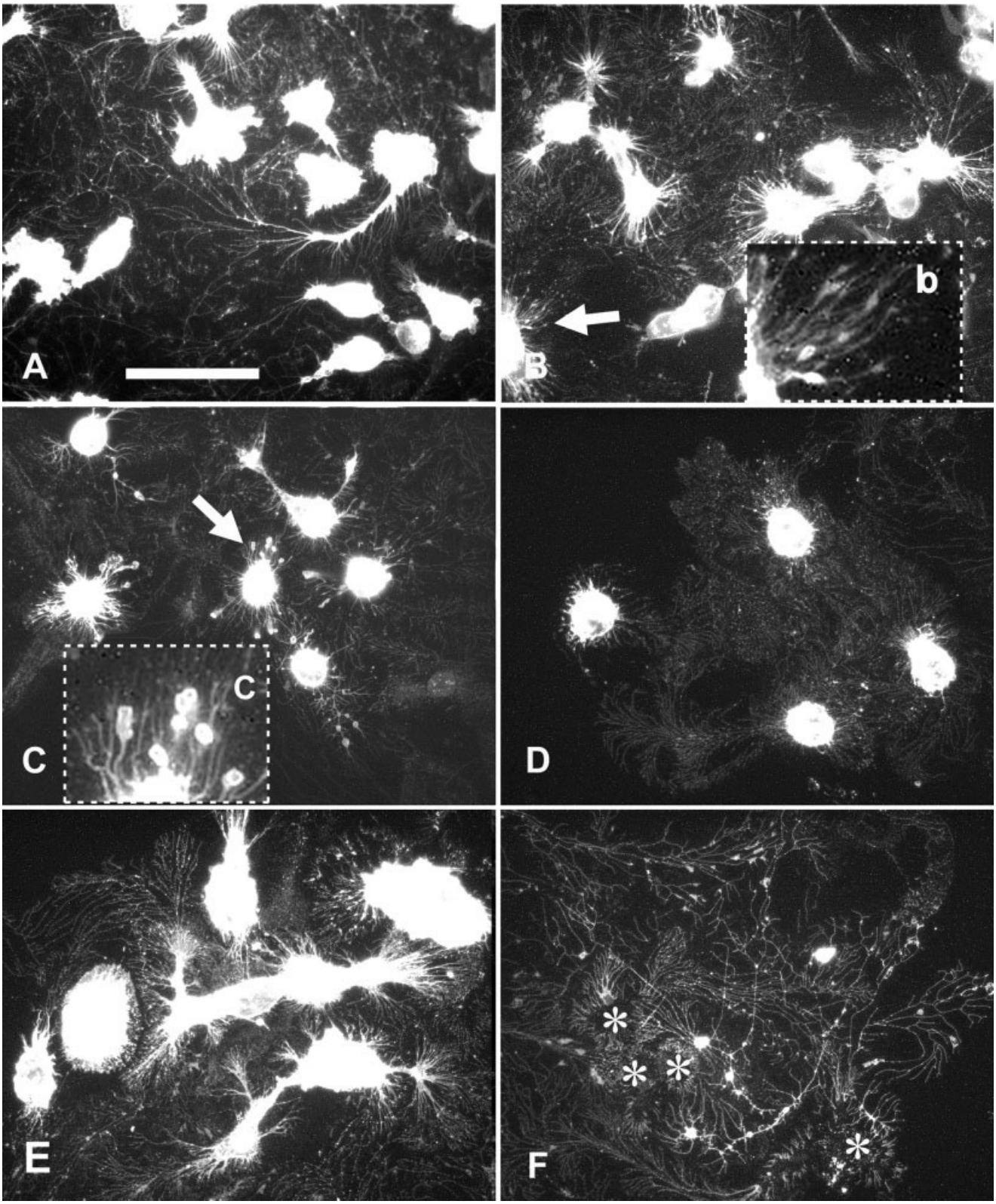

Figure 4.

TABLE II. Length of Fine Processes of Microglial Cell at Different Times After Stimulation With ATP (3 mM)★

	Con	30 min	1 hr	1.5 hr
N9	102 ± 12	97 ± 13	94 ± 17	92 ± 13
P	98 ± 16	92 ± 15	90 ± 12	91 ± 16

★The longest process was measured from a randomly selected cell and 150 cells were selected from one experiment. Four independent experiments were carried out. The data represent the mean ±SEM (μm). P, primary culture; N9, mouse microglial cell line. No significant changes were observed with respect to the control group, analysed by Student's *t*-test.

TABLE III. Length and Number of New Process of Microglial Cell at Different Times After Stimulation With ATP (3 mM)†

	30 min		1 hr		1.5 hr	
	L	N	L	N	L	N
N9	37 ± 6	38 ± 12	33 ± 5	32 ± 8	5 ± 3★	6 ± 3★
P	32 ± 5	35 ± 9	29 ± 6	31 ± 11	6 ± 2★	4 ± 3★

†The length of new process was measured from a randomly selected cell and 150 cells were selected from one experiment. Four independent experiments were carried out. The data represent the mean ± SEM (μm). P, primary culture; N9, mouse microglial cell line; L, length; N, number of the new processes.
★$P < 0.01$ with respect to 30 min group, analysed by Student's *t*-test.

TABLE IV. Length of Fine Processes of Microglial Cell at Different Times After Stimulation With Suramin Alone or Suramin and ATP★

		30 min		1 hr		1.5 hr	
	Con	S	S + A	S	S + A	S	S + A
N9	116 ± 13	114 ± 17	106 ± 16	112 ± 18	108 ± 21	103 ± 15	98 ± 21
P	108 ± 12	109 ± 14	121 ± 18	117 ± 15	109 ± 17	106 ± 13	99 ± 24

★In the control group, the cells were incubated in serum-free DMEM. The longest process was measured from a randomly selected cell and 150 cells were selected from one experiment, four independent experiments were carried out. The data represent the mean ± SEM (μm). S, suramin only (100 μM); S+A, suramin (100 μM) and ATP (3 mM); P, primary culture; N9, mouse microglial cell line. No significant changes were observed with respect to the control group, analysed by Student's *t*-test for two groups.

TABLE V. Cell Body Size of Microglial Cell at Different Times After Stimulation With ATP (3 mM)†

	Con	30 min	1 hr	2 hr
N9	46 ± 7	37 ± 8	28 ± 6★★	20 ± 3★★
P	47 ± 9	36 ± 6★	30 ± 7★★	22 ± 5★★

†The largest diameter was measured from a randomly selected cell and 150 cells were selected in one experiment, four independent experiments were carried out. Data represent the mean ± SEM (μm). P, primary culture; N9, mouse microglial cell line.
★$P < 0.05$, ★★$P < 0.01$ with respect to the control group, analysed by Student's *t*-test.

DISCUSSION

Microglial cells, a class of brain mononuclear phagocytes, are thought to be the principal immune cells resident to the CNS. Primitive stem cells, which give rise to microglial cells, are first seen in the periventricular regions during early embryonic development and eventually take up residence throughout the foetal brain (del Rio-Hortega, 1932; Ling, 1979). In the late foetal and early neonatal periods, maturing microglia differentiate from the amoeboid form into ramified forms, characteristic of the quiescent cells found in the adult nervous system (del Rio-Hortega, 1932). The ramified microglial cells will transform into the amoeboid-like cells after brain traumatic injury, inflammation and ischemia (Streit, 2002; Vilhardt, 2005). It was believed that the ramified microglial cells retract their processes and became amoeboid-like cells (del Rio-Hortega, 1932). Scanning electron microscopic studies in vitro showed that immunoactivators such as lipopolysaccharide and interferon-γ (IFN-γ) drove microglia to retract their processes (Giulian et al., 1995).

The ATP receptor (P2 receptor or purinoceptor) is the generic name for receptors that are activated by purines (e.g., ATP, ADP) and pyrimidines (e.g., UTP, UDP). It is now accepted that ATP receptors can be divided into two families: P2X and P2Y (Abbracchio and Burnstock, 1994; Ralevic and Burnstock, 1998). The P2X family ($P2X_1$–$P2X_7$) are ligand-gated ion channels. The P2Y family ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) is a member of the superfamily of G protein-coupled receptors. Pharmacological and physiological findings, as well as immunohistochemical evidence, suggest that microglial cells express P2 receptor protein molecules of the $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_2$, and $P2Y_{12}$ subtypes (Ferrari et al., 1996; Gao et al., 1999; Hide et al., 2000; Möller et al., 2000; Honda et al., 2001; Tsuda, et al., 2003, 2004; Xiang and Burnstock, 2005). Under normal conditions, the concentration of ATP is at the micromolar level in extracellular fluid, but at the millimolar level in the cytoplasm. After brain injury, the damaged cells from the loci of traumatic brain injury, inflammation or ischemia release ATP and UTP to activate the microglial cells via P2 receptors (Illes et al., 1996). Recently, with a novel technique, time-lapse two-photon imaging of green fluorescent protein-labeled microglia, Davalos et al. (2005) demonstrated that the fine termini of microglial processes are highly dynamic in the intact mouse cortex. On traumatic brain injury, microglial processes rapidly and autonomously converge on the site of injury without

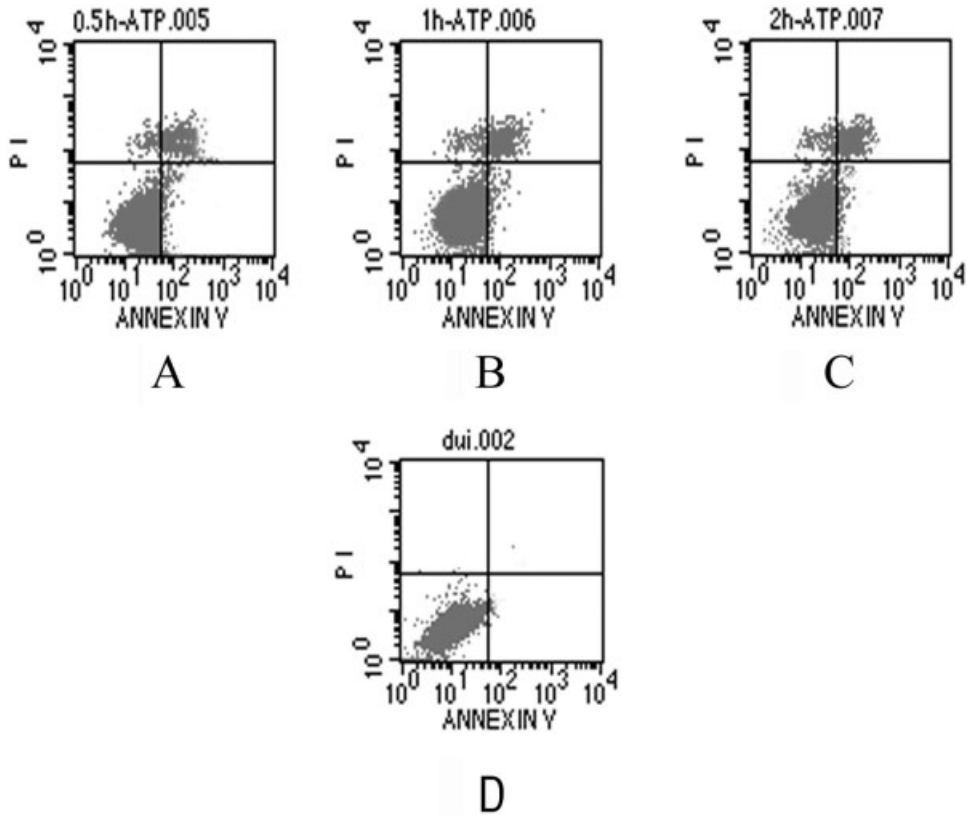

Fig. 5. The results of flow cytometry analysis of N9 microglial cells after stimulation with ATP (3 mM). **A–C:** Results of flow cytometry analysis of N9 microglial cells after stimulation with ATP for 0.5 hr, 1 hr, and 2 hr, respectively. **D:** Result from the control group.

TABLE VI. Percentages of Live, Apoptotic and Necrotic Cells in the Floating Population After Stimulation With ATP (3 mM) For Different Lengths of Time[†]

	Con	0.5 hr	1.0 hr	2.0 hr
Live cells	98 ± 1.2	78 ± 2.8★	70 ± 3.4★★	61 ± 1.8★★
Apoptotic cells	1.2 ± 1.0	8 ± 0.8★	14 ± 1.2★★	16 ± 1.8★★
Necrotic cells	0.8 ± 0.5	14 ± 1.8★	16 ± 2.3★★	23 ± 2.5★★

[†]Live cells were propidium iodide (PI)-negative and fluorescein isothiocyanate (FITC)-negative; apoptotic cells were PI-negative and FITC-positive; necrotic cells were PI-positive.

★$P < 0.05$, ★★$P < 0.01$ with respect to the control group, analysed by Student's *t*-test. Four individual experiments were carried out.

cell body movement, establishing a potential barrier between the healthy and injured tissue. This rapid chemotactic response can be mimicked by local injection of ATP and can be inhibited by the ATP-hydrolyzing enzyme apyrase or by blockers of G protein-coupled purinergic receptors and connexin channels, which are highly expressed in astrocytes. The baseline motility of microglial processes is also reduced significantly in the presence of apyrase and connexin channel inhibitors. Thus, extracellular ATP regulates microglial branch dynamics in the intact brain, and its release from the damaged tissue and surrounding astrocytes mediates a rapid microglial response toward injury.

In the present study we found that extracellular ATP may activate microglial cells and transform them from the ramified form to the amoeboid form. During this morphological transformation most of their fine processes did not retract. These results might suggest that the ramified microglial cells are transformed into the amoeboid-like cells in a similar way after activation by extracellular ATP, UTP and their derivatives released during traumatic brain injury, ischemia and inflammation. It would be interesting, although difficult to study this morphological transformation in vivo.

Many of the small bulbs were formed after stimulation with ATP. These bulbs were thought to be the results of retracted processes (del Rio-Hortega, 1932). Recently, however, another possibility has been raised, that these bulbs are vesicles formed from the plasma membrane of microglial cells after ATP exposure and containing IL-1β, release of which is enhanced by ATP (Bianco et al., 2005b).

In the present study 30 min after re-plating, the primary cultured microglial cells and N9 microglial cells began to show fine processes. Sixteen hours after re-plating, the primary cultured microglial cells and N9 microglial cells were already in the ramified resting state, as determined by their morphology. After ATP stimulation, the microglial cells became amoeboid-like cells and most of them floated in the culture medium. Flow cytometry analyses showed that many of these floating microglial cells were alive. Half of them could still recover to the

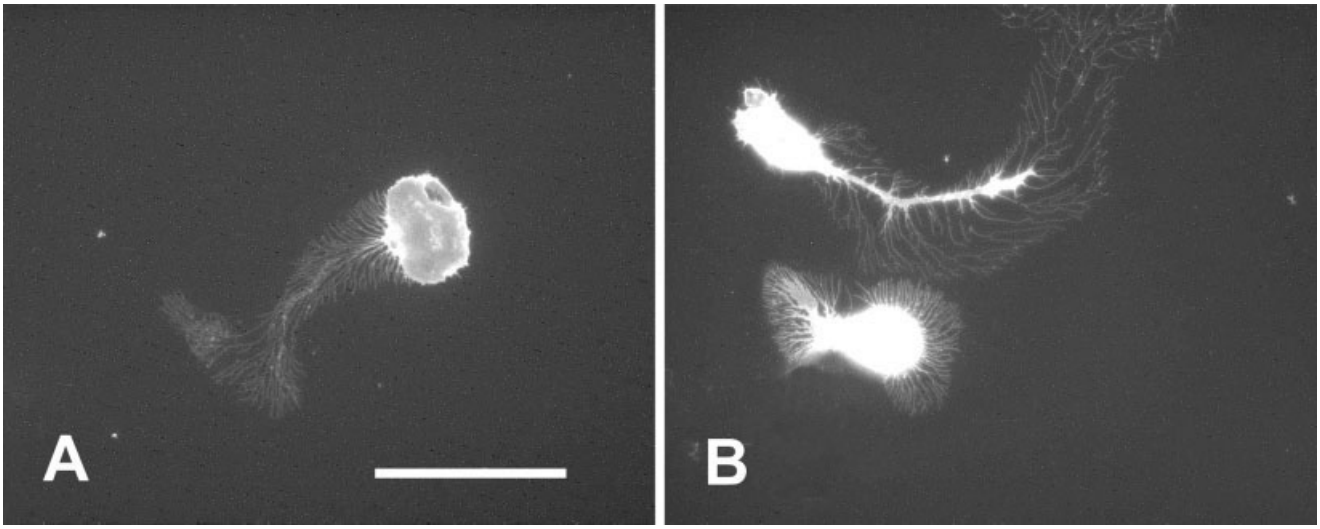

Fig. 6. Detached N9 microglial cells collected from the medium, re-cultured and stimulated with ATP (3 mM) shown by Biotin-IB4-streptavidin-FITC staining. **A:** Re-cultured for 6 hr. **B:** Re-cultured for 12 hr.

resting, ramified state 16–24 hr after removal of ATP from the culture medium. These results showed that N9 microglial cells and primary cultured rat microglial cells are good models for studying the transformation from the resting microglial cells to the amoeboid form.

Recently, CX43 expression and functional gap junctions have been described in macrophages/microglia. In vitro dye coupling studies with primary rat microglia confirmed that gap junctional communications existed in the microglial cells activated by tumour necrosis factor-α (TNF-α) or IFN-γ (Eugenin et al., 2001, 2003). The present study showed that the fine processes of the microglial cells as demonstrated by IB4 and Streptavidin-FITC staining were very long and complicated. A network of these processes of the microglial cells was clearly formed 24 hr or more after re-plating. These processes seemed to connect each other or with processes from other cell bodies (Fig. 1). This suggests that neighbouring microglial cells might communicate with each other via their fine processes. To examine this possibility, Lucifer Yellow was injected into N9 microglial cells. However, no neighbouring microglial cells were labeled, although the injected microglial cell was demonstrated clearly by Lucifer Yellow. These results implied that there was no gap-junctional communications between cultured microglial cells, at least in our culture conditions. This result is consistent with previous reports that few gap-junctional communications exist in resting primary microglial cells (Eugenin et al., 2001, 2003). It would be interesting to further study whether gap-junctional communication exists in these processes of microglial cells after activation by stimulators such as TNF-α, IFN-γ, or ATP.

In summary, novel morphological characteristics of N9 microglial cells and primary cultured rat microglial cells have been demonstrated by bitotin-IB4 and streptavidin-FITC staining in the present study. Numerous fine, long processes of the microglial cells formed a network. A dye coupling study did not show gap-junctional communication between neighbouring cells via the processes, at least in normal conditions. Most of the primary cultured microglial cells and N9 microglial cells send out their processes by 30 min in culture and a process network is well formed 16–24 hr after re-plating, at that time the morphological characteristics are similar with that of the resting state of the microglial cells. After being challenged by 3 mM ATP the microglial cells were activated, became amoeboid-like cells and finally floated in the cultured medium, but the complicated network of the processes remained. Flow cytometry analysis showed that the majority of these floating cells were alive, and could recover to the resting state after ATP was removed from the culture medium.

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