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European Journal of Pharmacology 399 (2000) 117–121

ejp

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Short communication

Neuroprotective activity of chemokines against *N*-methyl-D-aspartate or β-amyloid-induced toxicity in culture

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Received 14 April 2000; received in revised form 12 May 2000; accepted 16 May 2000

Abstract

We have examined the effect of various chemokines on neuronal toxicity in culture. In mixed cortical cultures, challenged with a brief pulse of *N*-methyl-D-aspartate (NMDA, 60 μM, 10 min), chemokines were either present for 2 h preceding the pulse or they were co-applied with NMDA and then kept in the medium for the following 20–24 h. Interleukin-8 (IL-8), regulated on activation of normal T cells expressed and secreted (RANTES) and macrophage/monocyte chemoattractant protein-1 (MCP-1), were neuroprotective under both conditions, whereas stromal cell-derived factor 1α (SDF-1α) was protective only when applied during and after the NMDA pulse. Mixed or pure neuronal cultures were also exposed for 48 h to a toxic fragment of the β-amyloid peptide (β-amyloid peptide-(25-35), 12.5 or 25 μM) in the absence or presence of chemokines. Among a number of chemokines, only RANTES was neuroprotective against β-amyloid peptide-(25-35)-induced neurotoxicity in both cultures. We conclude that activation of chemokine receptors differentially affects neuronal degeneration induced by excitotoxins or β-amyloid peptide in cortical cultures.

Keywords: Chemokine; *N*-methyl-D-aspartate (NMDA); β-amyloid; Cortical culture; Neuroprotection

1. Introduction

Chemokines constitute a family of chemotactic cytokines widely involved in the regulation of inflammation and in the development of the immune system (reviewed by Mennicken et al., 1999). Chemokines are subdivided into four subfamilies based on the number and location of cysteine residues in their primary structures. Members of the ''CXC'' or α subfamily, such as interleukin-8 (IL-8), stromal cell-derived factor 1α (SDF-1α), γ-interferon induced protein 10 (IP-10) and macrophage inflammatory protein-2 (MIP-2), are characterized by the presence of four cysteine residues, with the first two being separated by one amino acid. In the chemokines of the ''CC'' or β subfamily, such as regulated on activation of normal T cells expressed and secreted (RANTES), MIP-1α, macrophage/monocyte chemoattractant protein-1 (MCP-1), -3, and -5, the first two cysteines are next to one another; fractalkine belongs to the γ subfamily and has the first two cysteines separated by three amino acids; the δ subfamily includes lymphotactin, which possesses only two cysteines (Miller and Meucci, 1999). Chemokines interact with G-protein-coupled receptors named CXCR, CCR or CX3CR. Within the CXC subfamily, MIP-2, IP-10 and SDF-1α interact with CXCR2, -3 and -4, respectively, whereas IL-8 activates both CXCR1 and CXCR2. Most of the members of the CC subfamily are promiscuous and activate different CCRs. RANTES activates CCR1, -3, -4 and -5; MCP-1 activates CCR2, -4 and -9; MCP-3 activates CCR1, -2 and -3; MCP-5 activates CCR2; and MIP-1α activates CCR1, -4 and -5 (Mennicken et al., 1999; Miller and Meucci, 1999). How individual chemokine receptors transduce the extracellular signal into specific intracellular responses is unclear, although the activation of multiple transduction pathways has been reported (Miller and Meucci, 1999). A number of chemokine

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00367-8

receptors, including CXCR4, CCR3, CCR5, act as co-receptors for the human immunodeficiency virus (HIV) coat protein gp120, and their absence protects individuals otherwise at risk against HIV infection (reviewed by Clapham and Weiss, 1997). In the central nervous system (CNS), chemokines are mostly expressed by astrocytes and microglia, whereas their receptors are also present in neurons (Mennicken et al., 1999). How activation of chemokine receptors affects neuronal function under normal and pathological conditions is still unknown. Recently, Meucci et al. (1998) have shown that chemokines of different subfamilies, such as macrophage-derived chemokine (MDC), RANTES, SDF-1α and fractalkine, protect hippocampal neurons against gp120 neurotoxicity, suggesting a role for chemokine receptors in the pathophysiology of neurodegeneration associated with acquired immunodeficiency syndrome (AIDS). We were intrigued by the finding that the same chemokines are also protective against neuronal apoptosis induced by trophic deprivation (Meucci et al., 1999) and that knock-out mice lacking the CXCR4 receptor show profound alterations in cerebellar development and die perinatally (Zou et al., 1998). Thus, it appears that activation of certain chemokine receptors supports neuronal survival, independently of the presence of gp120 and is also required for proper CNS development (Mennicken et al., 1999). We now report the effect of different chemokines on neuronal toxicity induced by *N*-methyl-D-aspartate (NMDA) or β-amyloid peptide in culture.

2. Materials and methods

2.1. Mixed cortical cultures

Mixed cortical cell cultures containing both neurons and astrocytes were prepared from fetal mice at 14–16 days of gestation, as described previously (Bruno et al., 1995). In brief, dissociated cortical cells were plated on a layer of confluent astrocytes in minimal essential medium (MEM)-Eagle's salts (supplied glutamine free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, glutamine (2 mM), and glucose (final concentration 21 mM). After 3–5 days in vitro, non-neuronal cell division was halted by 1–3 days of exposure to 10 μM cytosine arabinoside. Mature cultures (13–14 days in vitro) were used for the experiments.

2.2. Pure cultures of cortical neurons

Cultures of pure cortical neurons were obtained from E15 rat embryos, as described by Copani et al. (1999). In brief, cells were mechanically dissociated and plated in Dulbecco's Modified Essential Medium (DMEM)/Ham's F12 (1:1) supplemented with 10 mg/ml bovine serum albumin, 10 μg/ml insulin, 100 μg/ml transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, 2 mM glutamine, 6 mg/ml glucose, 50 units/ml penicillin and 50 μg/ml streptomycin. Cytosine-β-D-arabinofuranoside (10 μM) was added to the cultures 18 h after plating and was present for 3 days. This method yields more than 99% pure neuronal cultures (Copani et al., 1999). Cultures at 8–10 days in vitro were used for the study of β-amyloid peptide toxicity.

2.3. Exposure of mixed cortical cultures to NMDA

Exposure to NMDA (60 μM for 10 min) was carried out in mixed cortical cultures at room temperature in a HEPES-buffered salt solution (Bruno et al., 1995). At the end of this incubation, cultures were extensively washed and then incubated at 37°C for the following 24 h in medium stock (MEM-Eagle's supplemented with 15.8 mM $NaHCO_3$ and glucose < 25 mM).

2.4. Exposure of mixed cortical cultures or pure neuronal cultures

β-amyloid peptide-(25-35) was solubilized in sterile water at a concentration of 2.5 mM and was stored frozen at −20°C for at least 1 week prior to use. Cultures were exposed to β-amyloid peptide-(25-35) (12.5 or 25 μM) for 48 h in the presence of 30 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 10 μM dizocilpine (MK-801). Under these conditions, β-amyloid peptide-(25-35) induced apoptotic neuronal death, followed by secondary necrosis (Copani et al., 1995). DNQX and MK-801 were not toxic per se when applied in combination in both mixed cortical cultures or pure neuronal cultures.

2.5. Assessment of in vitro neuronal injury

NMDA and β-amyloid peptide-(25-35) toxicity were estimated by counting dead neurons after Trypan blue staining, 20–24 h after the insult. In pure neuronal cultures, β-amyloid peptide-(25-35) toxicity was assessed by the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

2.6. Materials

Tissue culture reagents were from Sigma (Milano, Italy). NMDA, DNQX and MK-801 were purchased from Tocris (Bristol, UK). β-amyloid peptide-(25-35) was obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). Recombinant human IL-8, human SDF-1α, murine IP-10, murine or rat RANTES, rat MCP-1, murine MCP-3, murine MCP-5, murine MIP-1α, and rat MIP-2 were purchased from Pepro Tech EC (London, UK).

3. Results

3.1. Effect of chemokines on NMDA toxicity

A 10-min exposure of mixed cultures of mouse cortical cells to 60 μM NMDA killed about 60% of the neuronal cells (considered as 100% in Fig. 1). Chemokines belonging to the CXC (IL-8, SDF-1α, IP-10) or CC (RANTES, MCP-1, MCP-3, MCP-5, MIP-1α) subfamilies were either applied 2 h before and washed out prior to the NMDA pulse, or they were co-applied with NMDA and were present for the following 20–24 h.

In the pre-exposure paradigm, only IL-8 was neuroprotective in a wide range of concentrations (from 3 to 100 ng/ml; for expression in molarity, see legend of Fig 1).

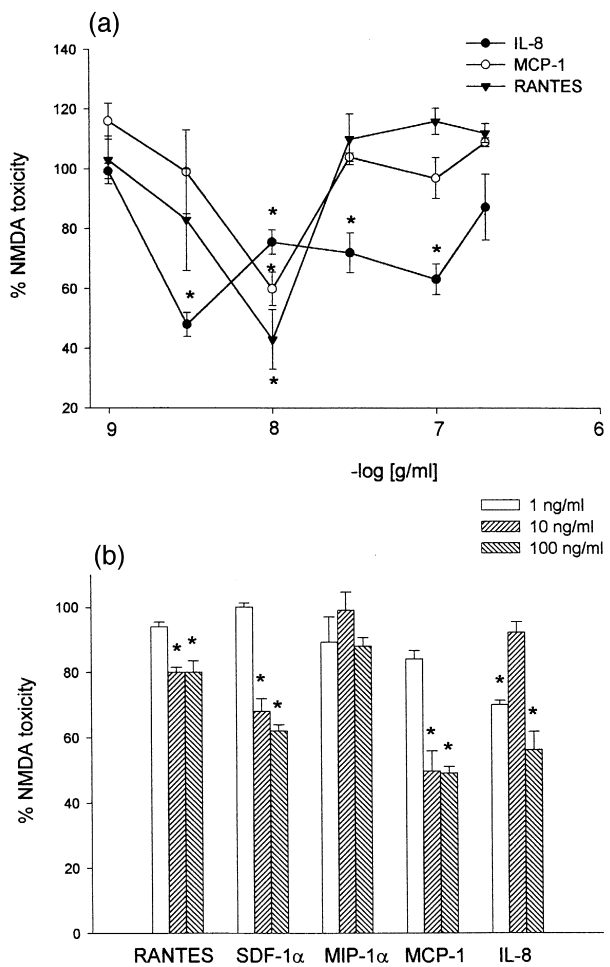

Fig. 1. (a) Concentration-dependent effect of IL-8, MCP-1 or mouse RANTES on NMDA toxicity in the pre-exposure paradigm. Chemokines were present in the medium of mixed cortical cultures during the 2 h, preceding the 10-min pulse with 60 μM NMDA. (b) Modulation of neuronal toxicity by chemokines co-applied with NMDA and present in the medium for the following 20–24 h. Values are means ± S.E.M. of 6–12 (a) or 6–9 (b) determinations. * $P < 0.01$ (One-way ANOVA + Fisher's PLSD) vs. NMDA alone. When expressed in molarity, 10 ng/ml of chemokines corresponds to: 1.25 nM for IL-8 and SDF-1α; 1.36 nM for IP10; 1.44 nM for MCP-5; 1.33 nM for MCP-3; 2.19 nM for MCP-1; and 1.22 nM for RANTES and MIP-1α.

Table 1
Effect of different chemokines on β-amyloid peptide toxicity in mixed cultures of cortical cells or in pure cultures of cortical neurons

% β-amyloid peptide-(25-35) toxicity			
Mixed cultures		Pure cultures	
IP-10, 10 ng/ml	105 ± 8.6	MCP-1, 100 ng/ml	104 ± 11
SDF-1α, 10 ng/ml	139 ± 11*	MIP-2, 50 ng/ml	89 ± 16
IL-8, 100 ng/ml	94 ± 8	MIP-2, 100 ng/ml	125 ± 21
MCP-1, 100 ng/ml	95 ± 8	Rat RANTES, 10 ng/ml	106 ± 17
MIP-1α, 100 ng/ml	128 ± 7.5	Rat RANTES, 100 ng/ml	56 ± 4.2*
Mouse RANTES, 10 ng/ml	58 ± 2.1*	Rat RANTES, 200 ng/ml	11 ± 1.5*
		SDF-1α, 10 ng/ml	97 ± 6.3
		IL-8, 100 ng/ml	103 ± 5.6

Values are means ± S.E.M. of 6 (mixed cultures) or 8–16 (pure cultures) determinations. β-amyloid peptide-(25-35) was applied for 48 h in the presence of 10 μM MK-801 and 30 μM DNQX at concentrations of 12.5 μM (mixed cultures) or 25 μM (pure cultures). β-amyloid peptide-(25-35) toxicity was assessed by Trypan blue staining in mixed cultures and by the MTT assay in pure neuronal cultures.

* $P < 0.01$ (ANOVA + Bonferroni's t test) vs. β-amyloid peptide-(25-35).

Inverse bell-shaped concentration–response curves were obtained for mouse RANTES or MCP-1, with both chemokines being protective only at 10 ng/ml (Fig. 1a). Similarly, IP-10 was slightly neuroprotective at 10 ng/ml (77 ± 6.5% of NMDA toxicity, $n = 12$, $P < 0.05$). SDF-1α and MIP-1α(1–200 ng/ml) did not affect NMDA toxicity; MCP-3 and MCP-5 were only tested at concentrations of 100 ng/ml and were inactive (not shown).

Results were different when cultures were exposed to chemokines both during and after the NMDA pulse. Under these conditions, mouse RANTES was slightly neuroprotective at 10 and 100 ng/ml, and substantial neuroprotection was observed with MCP-1, SDF-1α and IL-8. The latter, however, was effective at 1 and 100, but not at 10 ng/ml, for unexplained reasons. MIP-1α was inactive (Fig. 1b).

3.2. Effect of chemokines on β-amyloid peptide toxicity

Chemokines were co-applied with β-amyloid peptide-(25-35) to mixed mouse cortical cultures or pure cultures of rat cortical neurons and were present in the medium for the duration of β-amyloid peptide-(25-35) exposure (48 h). In both culture models, only RANTES attenuated β-amyloid peptide-(25-35)-induced neurotoxicity. Neuroprotection was observed with 10 ng/ml of mouse RANTES in mixed cultures, and with 100 or 200 ng/ml of rat RANTES in pure cultures (Table 1). SDF-1α amplified neurotoxicity in mixed cultures (but not in pure neuronal cultures), whereas all other chemokines were inactive (Table 1).

4. Discussion

Chemokine receptors, including CXCR2, CXCR4, CCR1, CCR3 and CCR5, have been detected in the CNS,

with at least CXCR4, CCR3 and CCR5 being expressed in cortical neurons (Klein et al., 1999). A role for chemokine receptors in neuronal death/survival is suggested by the expression of CXCR2 in dystrophic neurites surrounding amyloid plaques (Xia et al., 1997), and by the enhanced expression of CCR5 after injection of NMDA in the rat hippocampus (Galasso et al., 1998). The present results provide the first evidence for a neuroprotective activity of chemokines against NMDA or β-amyloid toxicity. The action of chemokines, however, was not uniform, but varied, in relation to the extracellular insult and to the manner of exposure to chemokines. In cultures treated with chemokines prior to the NMDA pulse, IL-8 was neuroprotective in a wide range of concentrations, whereas other chemokines were only active at 10 ng/ml. Because CXCR1 is not found in the CNS (Horuk et al., 1997), activation of CXCR2 might mediate neuroprotection by IL-8. The specificity of IL-8 for NMDA vs. βAP toxicity and its efficacy in the pre-exposure paradigm suggests that activation of IL-8 receptors negatively modulates NMDA receptor function. This is a likely possibility, because many G-protein-coupled receptors are functionally coupled to NMDA receptors. The bell-shaped concentration–response curve observed for RANTES and MCP-1 suggests that these chemokines activate high-affinity receptors that negatively modulate NMDA toxicity, but also low affinity receptors that subserve an opposite function. The use of receptor subtype-selective antagonists is needed to examine this possibility. When cultures were exposed to chemokines, both during and after the NMDA pulse, SDF-1α became neuroprotective and RANTES and MCP-1 were also protective at 100 ng/ml. This more generic form of neuroprotection suggests the existence of multiple chemokine receptors that control events triggered during and/or after NMDA exposure. Based on electrophysiological data, it has been hypothesized that some chemokine receptors are presynaptically located and function by inhibiting glutamate release (Meucci et al., 1998). This may explain our results because an enhanced release of glutamate during and after the NMDA pulse participates in the development of neuronal death in culture (Monyer et al., 1992). Interestingly, RANTES was protective against β-amyloid peptide toxicity (see also Kornecook et al., 1999), under conditions in which endogenous activation of ionotropic glutamate receptors was prevented by the presence of MK-801 and DNQX. All other chemokines were inactive with the exception of SDF-1α, which slightly enhanced NMDA toxicity. How can this be reconciled with the protective activity of SDF-1α against NMDA toxicity is unclear at present. We could also examine the effect of different chemokines on β-amyloid peptide toxicity in pure cultures of rat cortical neurons, which are virtually devoid of astrocytes (Copani et al., 1999). These cultures could be used because they are sensitive to the toxic action of β-amyloid peptide as early as 8–10 days after plating, but are only partially sensitive to NMDA. RANTES was again the only chemokine showing neuroprotective activity against β-amyloid peptide toxicity in pure neuronal cultures, although higher concentrations were required than in mixed cortical cultures. This might reflect a lower potency of rat RANTES vs. mouse RANTES or a contribution of astrocytes to the action of this chemokine. Among the receptors activated by RANTES, CCR3 is a candidate for neuroprotection against β-amyloid peptide toxicity, because CCR1, -4 and -5 are recruited by MCP-1 and MIP-1α, which were inactive. This, however, can only be addressed by using subtype-selective antagonists or cultures prepared from knockout mice lacking individual chemokine receptors.

In conclusion, our data suggest that chemokines released during neuroinflammation not only regulate the traffic of leukocytes and monocytes in brain tissue, but can also interfere with the development of neuronal degeneration in response to endogenous excitotoxins or to aggregates of β-amyloid peptide present in amyloid plaques. To what extent is this relevant for the pathophysiology of neuronal death and be targeted by pharmacological treatments remains to be established.

References

Bruno, V., Copani, A., Knopfel, T., Kuhn, R., Casabona, G., Dell'Albani, P., Condorelli, D.F., Nicoletti, F., 1995. Activation of metabotropic glutamate receptors coupled to inositol phospholipid hydrolysis amplifies NMDA-induced neuronal degeneration in cultured cortical cells. Neuropharmacology 34, 1089–1098.

Clapham, P.R., Weiss, R.A., 1997. Immunodeficiency viruses. Spoilt for choice of co-receptors. Nature 388, 230–231.

Copani, A., Bruno, V., Battaglia, G., Leanza, G., Pellitteri, R., Russo, A., Stanzani, S., Nicoletti, F., 1995. Activation of metabotropic glutamate receptors protects cultured neurons against apoptosis induced by beta-amyloid peptide. Mol. Pharmacol. 47, 890–897.

Copani, A., Condorelli, D.F., Caruso, A., Vancheri, C., Sala, A., Giuffrida-Stella, A.M., Canonico, P.L., Nicoletti, F., Sortino, M.A., 1999. Mitotic signaling by β-amyloid causes neuronal death. FASEB J. 13, 2225–2234.

Galasso, J.M., Harrison, J.K., Silverstein, F.S., 1998. Excitotoxic brain injury stimulates expression of the chemokine receptor CCR5 in neonatal rats. Am. J. Pathol. 153, 1631–1640.

Horuk, R., Martin, A.W., Wang, Z., Schweitzer, L., Gerassimides, A., Guo, H., Lu, Z., Hesselgesser, J., Perez, H.D., Kim, J., Parker, J., Hadley, T.J., Peiper, S.C., 1997. Expression of chemokine receptors by subsets of neurons in the central nervous system. J. Immunol. 158, 2882–2890.

Klein, R.S., Williams, K.C., Alvarez Hernandez, X., Westmoreland, S., Force, T., Lackner, A.A., Luster, A.D., 1999. Chemokine receptor expression and signaling in macaque and human fetal neurons and astrocytes: implications for the neuropathogenesis of AIDS. J. Immunol. 163, 1636–1646.

Kornecook, T.J., Bastianetto, S., Quirion, R., 1999. Effect of chemokines on β-amyloid-induced toxicity, Soc. Neurosci. Abs. 25, 277.1.

Mennicken, F., Maki, R.B., de Souza, E., Quirion, R., 1999. Chemokines and chemokine receptors in the CNS: a possible role in neuroinflammation and patterning. Trends Pharmacol. Sci. 20, 73–78.

Meucci, O., Fatatis, A., Simen, A.A., Bushell, T.J., Grey, P.W., Miller, R.J., 1998. Chemokines regulates hippocampal neuronal signaling and gp120 neurotoxicity. Proc. Natl. Acad. Sci. U. S. A. 95, 14500–14505.

Miller, R.J., Meucci, O., 1999. AIDS and the brain: is there a chemokine connection? Trends Neurosci. 22, 471–479.

Monyer, H., Giffard, R.G., Hartley, D.M., Dugan, L.L., Goldberg, M.P., Choi, D.W., 1992. Oxygen and glucose deprivation-induced neuronal injury in cortical cell cultures is reduced by tetanus toxin. Neuron 8, 967–973.

Xia, M., Qin, S., McNamara, M., Mackay, C., Hyman, B.T., 1997. Interleukin-8 receptor B immunoreactivity in brain and neuritic plaques of Alzheimer's disease. Am. J. Pathol. 150, 1267–1274.

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., Littman, D.R., 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393, 595–599.