Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 337 (2005) 271–274
BBRC
www.elsevier.com/locate/ybbrc

Role of epiregulin in peptidoglycan-induced proinflammatory cytokine production by antigen presenting cells

Shigeru Sugiyama, Kazuhiko Nakabayashi, Iwai Baba, Takehiko Sasazuki, Senji Shirasawa *

Department of Pathology, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjyuku-ku, Tokyo 162-8655, Japan

Received 3 September 2005
Available online 16 September 2005

Abstract

We have previously found that epiregulin, a member of epidermal growth factor superfamily, is involved in proinflammatory cytokine production in bone marrow-derived macrophages. In this report, to further assess the role of epiregulin in innate immunity, we measured IL-6 production levels upon lipopolysaccharide and peptidoglycan stimulation in antigen presenting cells including macrophages and dendritic cells. Our analyses using epiregulin-deficient mice with mixed and inbred genetic backgrounds revealed that epiregulin deficiency results in the reduction of IL-6 production levels in both cell types upon peptidoglycan stimulation, and that the extent of this reduction is more evident under the BALB/c background compared with the C57BL/6J background. These results indicated that epiregulin may have a critical role in the regulation of peptidoglycan-mediated proinflammatory cytokine production in antigen presenting cells and innate immunity.

Keywords: Epiregulin; Peptidoglycan; Proinflammatory cytokine; Antigen presenting cells; Innate immunity

Epiregulin (EP) is one of the ligands for the epidermal growth factor receptor (EGFR) [1], originally purified from conditioned medium of the fibroblast-derived tumor cell line NIH3T3/T7 [2], and subsequently characterized to be an autocrine growth factor in keratinocytes in vitro [3]. Recent further studies revealed that EP functions in cellular signaling pathways in a wider variety of cell types [3–7]. EP has been shown to act as a major autocrine/paracrine factor in the differentiation of vascular smooth muscle cells [4,5], and as one of the paracrine mediators that propagate luteinizing hormone signals in the ovarian follicle [6]. However, EP functions in vivo still seem to be largely unknown. We independently identified *EP* as a gene up-regulated in cancer cells and demonstrated that its overexpression enhances cellular tumorigenicity in nude mice [7]. We also showed that, like many other EGF family members [9–11], EP exists as a membrane-bound form and a mature secreted form that is produced by the ectodomain shedding of the membrane-bound form [7,8].

To elucidate the physiological functions of EP, we established EP-deficient ($EP^{-/-}$) mice using gene targeting techniques. Interestingly, $EP^{-/-}$ mice showed atopy-like dermatitis [8]. Detailed analyses of the $EP^{-/-}$ mice revealed that EP plays essential roles in immune/inflammatory-related responses in keratinocytes and macrophages at the barrier from the outside milieu, and that secreted and membrane-bound forms of EP have distinct functions.

The secreted form of EP is involved in the proper regulation of IL-18 in karatinocytes, and the membrane-bound form of EP is involved in the proinflammatory cytokine production in response to Toll-like receptor (TLR) agonists in tissue resident macrophages [8].

During these analyses, *EP* was found to be highly expressed not only in epidermal cells but also in antigen presenting cells (APCs) that are present in secondary lymphatic tissues. Here, we characterized a critical role of EP in APCs in innate immune responses. Comparison of $EP^{-/-}$ and wild-type derived APCs in response to TLR

* Corresponding author. Fax: +81 3 3202 7364.
E-mail address: sshirasawa@ri.imcj.go.jp (S. Shirasawa).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.050

agonists revealed that EP has a pivotal role in peptidoglycan-induced proinflammatory cytokine production.

Materials and methods

Generation of the $EP^{-/-}$ lines with inbred genetic backgrounds. Male F1 hybrid mice (129/Sv × C57BL/6J) heterozygous for the *EP*-knockout allele [8] were mated with BALB/c or C57BL/6J female mice (6–8 weeks old). Male offspring heterozygous for the *EP*-knockout allele were backcrossed with BALB/c or C57BL/6J. After the backcross mating was repeated for eight generations, heterozygous offspring were intercrossed to obtain $EP^{-/-}$ mice. In this study, the original $EP^{-/-}$ mice with the mixed genetic background is referred to as $EP^{-/-}$ (129/Sv × C57BL/6J), and thenewly established inbred $EP^{-/-}$ lines as $EP^{-/-}$ (BALB/c) and $EP^{-/-}$ (C57BL/6J).

Preparation of bone marrow-derived macrophages and dendritic cells. Bone marrow-derived macrophages and dendritic cells were prepared using the culture conditions described previously [12,13]. Briefly, to induce the proliferation of macrophages, bone marrow cells were plated in 10-cm plastic plates with 10% fetal calf serum (FCS) in RPMI1640 supplemented with 100 ng/ml of recombinant murine M-CSF (R&D). On day 7, adherent cells were collected with a scraper. To induce the proliferation of dendritic cells, bone marrow cells were plated in 10-cm plates with 10% FCS in RPMI1640 supplemented with 10 ng/ml of recombinant murine GM-CSF (PEPROTEC). On day 6, loosely adherent cells were collected by gentle pipetting. The collected macrophages and dendritic cells were subjected to cytokine production assays.

Cell staining and flow cytometry. Single-cell suspensions were incubated at 2×10^5 cells/100 μl on ice in the staining buffer (phosphate-buffered saline containing 2.5% FCS and 0.01% NaN_3) with monoclonal antibodies for 15 min [12]. The antibodies used are anti-CD40, anti-CD11b, and anti-CD11c (BD Pharmingen). Stained cells were analyzed using a FACS Caliber and the Cell Quests software (Becton–Dickinson).

Measurement of TLR agonist-induced IL-6 production levels. Bone marrow-derived macrophages or dendritic cells were cultured in 24-well plates (5×10^5 per well) for 24 h and stimulated with lipopolysaccharide (LPS) (1 μg/ml) or peptidoglycan (PGN) (10 μg/ml). After 24 h incubation, the culture supernatants were subjected to ELISA kit (BioSource).

Results

We measured IL-6 production levels in bone marrow-derived dendritic cells (BMDCs) in response to LPS and PGN. Upon PGN stimulation, the IL-6 level was remarkably lower in the BMDCs derived from $EP^{-/-}$ (129/Sv × C57BL/6J) compared with the control BMDCs derived from wild-type mice (Fig. 1). However, there was no significant difference in the IL-6 levels between the $EP^{-/-}$ and the wild-type derived BMDCs upon LPS stimulation.

To assess the possibility of genetic backgrounds interfering with this difference, we established $EP^{-/-}$ mice with inbred genetic backgrounds, BALB/c and C57BL/6J, through eight generations of successive backcross matings. First, we measured the surface expression levels of CD40, a co-stimulatory molecule, in bone marrow-derived macrophages (BMDMs) and BMDCs in response to LPS and PGN. The CD40 levels were similar between the $EP^{-/-}$-derived and the corresponding wild-type derived cells upon both LPS and PGN stimulation under both inbred genetic backgrounds (Figs. 2A and B). These results confirm our previous finding from the analyses of

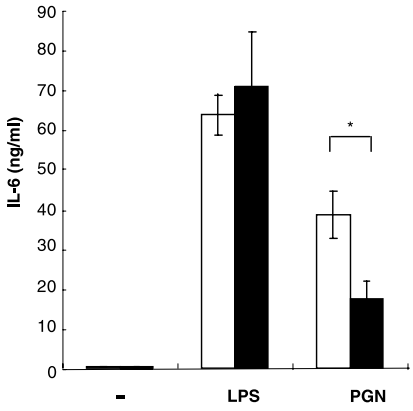

Fig. 1. TLR agonist-induced IL-6 production by BMDCs derived from $EP^{-/-}$ (129/Sv × C57BL/6J). IL-6 levels were measured without stimulation (−), and with LPS and PGN stimulation in BMDCs derived from $EP^{-/-}$ (129/Sv × C57BL/6J) mice and wild-type control mice with the same mixed genetic background. Data shown are means ± SD of triplicates in one experiment. Similar results were obtained in three independent experiments. Open bars, wild-type mice; solid bars, $EP^{-/-}$ mice. The statistical difference was determined by Student's *t* test. $*p < 0.05$.

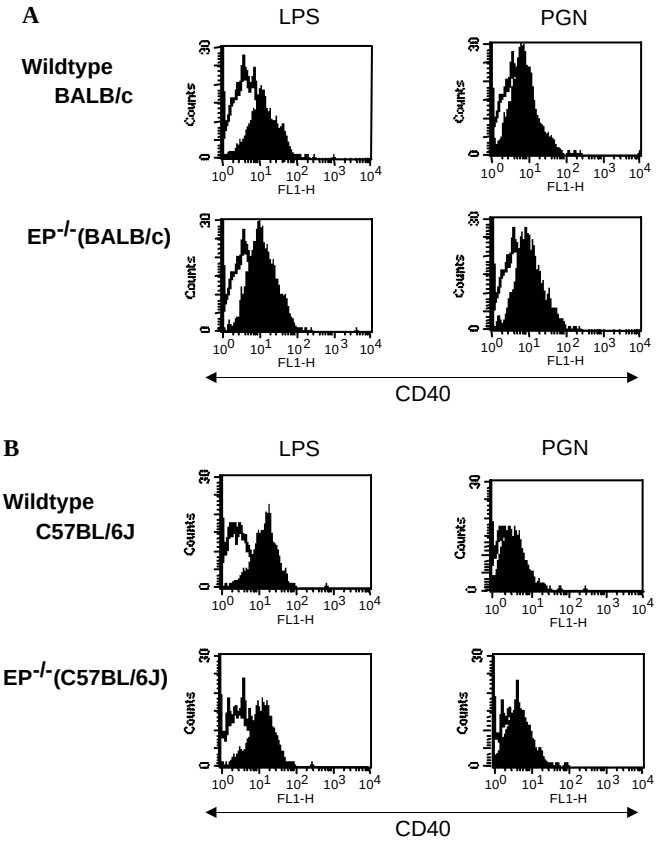

Fig. 2. TLR agonist-induced surface expression of CD40 by BMDMs measured by fluorescence-activated cell sorting (FACS) analysis. (A) $EP^{-/-}$ (BALB/c) and its wild-type control. (B) $EP^{-/-}$ (C57BL/6J) and its wild-type control. Experiments were repeated three times in triplicates, and similar results were obtained. Surface expression levels of CD40 in BMDCs were also similar between the inbred $EP^{-/-}$ lines and their wild-type controls (data not shown).

$EP^{-/-}$ (129/Sv × C57BL/6J) that EP-deficiency does not affect the surface expression levels of co-stimulatory molecules [8].

Next, we measured IL-6 production levels in BMDMs and BMDCs derived from the two inbred $EP^{-/-}$ lines and the corresponding wild-type control littermates. Upon LPS stimulation, in both cell types and under both genetic backgrounds tested, no significant difference was observed in the IL-6 production levels between $EP^{-/-}$-derived and wild-type derived cells (Figs. 3A–D). Upon PGN stimulation, the statistically significant reduction of IL-6 production levels in $EP^{-/-}$-derived cells was observed in both BMDMs and BMDCs under the BALB/c background (Figs. 3A and B), whereas it was observed only in BMDMs under the C57BL/6J background (Figs. 3C and D). In BMDCs derived from $EP^{-/-}$ (C57BL/6J), the IL-6 production levels were consistently lower compared with the wild-type control upon PGN stimulation; however, the difference was not statistically significant (Fig. 1D). These results, together, indicated that the PGN-induced IL-6 production by APCs is reduced due to epiregulin-deficiency and that the reduction is more evident under the BALB/c background compared with the C57BL/6J background.

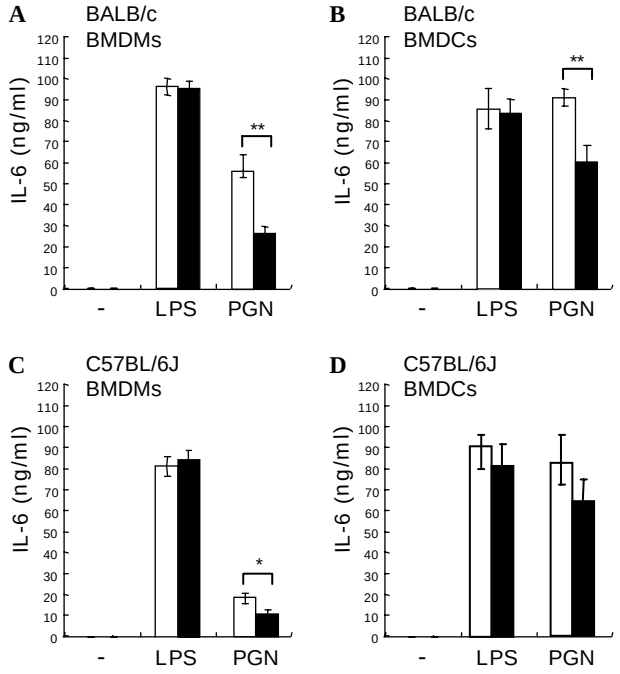

Fig. 3. TLR agonist-induced IL-6 production by antigen presenting cells derived from inbred $EP^{-/-}$ lines. (A) BMDMs derived from wild-type and $EP^{-/-}$ BALB/c. (B) BMDCs derived from wild-type and $EP^{-/-}$ BALB/c. (C) BMDMs derived from wild-type and $EP^{-/-}$ C57BL/6J. (D) BMDCs derived from wild-type and $EP^{-/-}$ C57BL/6J. Data shown are means ± SD of triplicates in one experiment. Similar results were obtained in three independent experiments. Open bars, wild-type mice; solid bars, $EP^{-/-}$ mice. The statistical differences were determined by Student's *t* test. $*p < 0.05$; $**p < 0.005$.

Discussion

Our findings from this and previous studies demonstrated the critical role of EP in the PGN-induced proinflammatory cytokine production by APCs. Recently, the proteolytically released carboxy-terminal fragments of two EGF family members, HB-EGF and Neuregulin-1, are reported to be translocated into the nucleus, culminating in transducing cellular signals [14,15]. Therefore, a possibility exists that the carboxy-terminal fragment of EP may act in a similar manner in the PGN-induced signaling. Addition of the soluble form of EP (sEP) or a EGFR blocker did not affect the PGN-induced IL-6 production levels in $EP^{-/-}$ and wild-type BMDMs (data not shown), indicating that the membrane-anchored form, but not sEP, will be involved in the PGN-mediated signaling pathway(s).

We chose BALB/c and C57BL/6J as inbred genetic backgrounds to carry the EP knockout allele homozygously, because these strains are known to express different immune responses due to polymorphisms in immune-related genes. In our previous analysis on $EP^{-/-}$ (129/Sv × C57BL/6J), proinflammatory cytokine production levels were remarkably reduced in the $EP^{-/-}$ macrophages compared with the wild-type control not only on PGN but also on LPS stimulation [8]. However, no significant difference in the LPS-induced IL-6 production was observed between the macrophages derived from $EP^{-/-}$ (BALB/c) and $EP^{-/-}$ (C57BL/6J), suggesting that the reduction of LPS-induced IL-6 production observed in BMDMs from $EP^{-/-}$ (129/Sv × C57BL/6J) [8] might be due to genetic polymorphisms in the 129/Sv strain.

The PGN-induced IL-6 production levels in $EP^{-/-}$ mice tended to be more remarkably reduced under the BALB/c background than the C57BL/6J background. It is known that BALB/c and C57BL/6J are biased towards Th2 and Th1 differentiation, respectively, and that BALB/c shows higher serum IgE levels compared with C57BL/6J [16]. Therefore, $EP^{-/-}$ (BALB/c) mice will be a useful model to further elucidate EP functions in the PGN signaling, innate immunity, and allergic inflammation.

Acknowledgment

This work was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] M. Shelly, R. Pinkas-Kramarski, B.C. Guarino, H. Waterman, L.M. Wang, L. Lyass, M. Alimandi, A. Kuo, S.S. Bacus, J.H. Pierce, Epiregulin is a potent pan-ErbB ligand that preferentially activates heterodimeric receptor complexes, J. Biol. Chem. 273 (1998) 10496–10505.

[2] H. Toyoda, T. Komurasaki, D. Uchida, Y. Takayama, T. Isobe, T. Okuyama, K. Hanada, Epiregulin: a novel epidermal growth factor with mitogenic activity for rat primary hepatocytes, J. Biol. Chem. 270 (1995) 7495–7500.

[3] Y. Shirakata, T. Komurasaki, H. Toyoda, Y. Hanakawa, K. Yamasaki, S. Tokumaru, K. Sayama, K. Hashimoto, Epiregulin, a novel member of the epidermal growth factor family, is an autocrine growth factor in normal human keratinocytes, J. Biol. Chem. 275 (2000) 5748–5753.
[4] D.S. Taylor, X. Cheng, J.E. Pawlowski, A.R. Wallace, P. Ferrer, C.J. Molloy, Epiregulin is a potent vascular smooth muscle cell-derived mitogen induced by angiotensin II, endothelin-1, and thrombin, Proc. Natl. Acad. Sci. USA 96 (1999) 1633–1638.
[5] M. Takahashi, K. Hayashi, K. Yoshida, Y. Ohkawa, T. Komurasaki, A. Kitabatake, A. Ogawa, W. Shida, M. Yano, M. Monden, Epiregulin as a major autocrine/paracrine factor released from ERK- and p38MAPK-activated vascular smooth muscle cells, Circulation 108 (2003) 2524–2529.
[6] J.Y. Park, Y.Q.M. Ariga, E. Law, S.L.C. Jin, M. Conti, EGF-like growth factors as mediators of LH action in the ovulatory follicle, Science 303 (2004) 682–684.
[7] I. Baba, S. Shirasawa, R. Iwamoto, K. Okumura, T. Tsunoda, M. Nishioka, K. Fukuyama, K. Yamamoto, E. Mekada, T. Sasazuki, Involvement of deregulated epiregulin expression in tumorigenesis in vivo through activated Ki-Ras signaling pathway in human colon cancer cells, Cancer Res. 60 (2000) 6886–6889.
[8] S. Shirasawa, S. Sugiyama, I. Baba, J. Inokuchi, S. Sekine, K. Ogino, Y. Kawamura, T. Dohi, M. Fujimoto, T. Sasazuki, Dermatitis due to epiregulin deficiency and a critical role of epiregulin in immune-related responses of keratinocyte and macrophage, Proc. Natl. Acad. Sci. USA 101 (2004) 13921–13926.
[9] J. Massagué, A. Pandiella, Membrane-anchored growth factors, Annu. Rev. Biochem. 62 (1993) 515–541.
[10] S.W. Sunnarborg, C.L. Hinkle, M. Stevenson, W.E. Russell, C.S. Raska, J.J. Peschon, B.J. Castner, M.J. Gerhart, R.J. Paxton, R.A. Black, Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability, J. Biol. Chem. 277 (2002) 12838–12845.
[11] R. Iwamoto, E. Mekada, Heparin-binding EGF-like growth factor: a juxtacrine growth factor, Cytokine Growth Factor Rev. 11 (2000) 335–344.
[12] Y. Nagai, S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, K. Miyake, Essential role of MD-2 in LPS responsiveness and TLR4 distribution, Nat. Immunol. 3 (2002) 667–672.
[13] K. Inaba, M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, R.M. Steinman, Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor, J. Exp. Med. 176 (1992) 1693–1702.
[14] D. Nanba, A. Mammoto, K. Hashimoto, S. Higashiyama, Proteolytic release of the carboxy-terminal fragment of proHB-EGF causes nuclear export of PLZF, J. Cell Biol. 163 (2003) 489–502.
[15] J. Bao, D. Wolpowitz, L.W. Role, D.A. Talmage, Back signaling by the Nrg-1 intracellular domain, J. Cell Biol. 161 (2003) 1133–1141.
[16] J.D. Gorham, M.L. Guler, R.G. Steen, A.J. Mackey, M.J. Daly, K. Frederick, W.F. Dietrich, K.M. Murphy, Genetic mapping of a murine locus controlling development of T helper 1/T helper 2 type responses, Proc. Natl. Acad. Sci. USA 93 (1996) 12467–12472.