Tumor-Secreted Lactic Acid Promotes IL-23/IL-17 Proinflammatory Pathway

Hiroaki Shime, Masahiko Yabu, Takashi Akazawa, Ken Kodama, Misako Matsumoto, Tsukasa Seya and Norimitsu Inoue

J Immunol 2008; 180:7175-7183; doi: 10.4049/jimmunol.180.11.7175
http://www.jimmunol.org/content/180/11/7175

References
This article cites 44 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/180/11/7175.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Tumor-Secreted Lactic Acid Promotes IL-23/IL-17 Proinflammatory Pathway

Hiroaki Shime,* Masahiko Yabu,† Takashi Akazawa,* Ken Kodama,‡ Misako Matsumoto,‡ Tsukasa Seya,‡ and Norimitsu Inoue2*

IL-23 is a proinflammatory cytokine consisting of a p19 subunit and a p40 subunit that is shared with IL-12. IL-23 is overexpressed in and around tumor tissues, where it induces local inflammation and promotes tumor development. Many tumor cells produce large amounts of lactic acid by altering their glucose metabolism. In this study, we show that lactic acid secreted by tumor cells enhances the transcription of IL-23p19 and IL-23 production in monocytes/macrophages and in tumor-infiltrating immune cells that are stimulated with TLR2 and 4 ligands. DNA elements responsible for this enhancing activity of lactic acid were detected in a 2.7-kb 5′-flanking region of the human IL-23p19 gene. The effect of lactic acid was strictly regulated by extracellular pH. Furthermore, by inducing IL-23 overproduction, lactic acid facilitated the Ag-dependent secretion of proinflammatory cytokine IL-17 but not IFN-γ by TLR ligand-stimulated mouse splenocytes. Interestingly, this effect was observed even in the absence of TLR ligand stimulation. These results suggest that rather than just being a terminal metabolite, lactic acid is a proinflammatory mediator that is secreted by tumor cells to activate the IL-23/IL-17 proinflammatory pathway but not the Th1 pathway. Targeting the lactic acid-induced proinflammatory response may be a useful approach for treating cancer.


*Department of Molecular Genetics and †Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan; and ‡Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan.

Received for publication May 23, 2007. Accepted for publication March 23, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by a grant from the Cooperative Link of Unique Science and Technology for Economic Revitalization promoted by the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science, and Technology.

2 Address correspondence and reprint requests to Dr. Norimitsu Inoue, Department of Molecular Genetics, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari-ku, Osaka 537-8511, Japan. E-mail address: inoue-no@mc.pref.osaka.jp

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

www.jimmunol.org
factors that might modulate the production of IL-23 by monocytes/macrophages stimulated with TLR ligands. In this study, we show that lactic acid secreted from tumor cells up-regulates TLR signal-dependent transcription of the IL-23p19 subunit in human and mouse monocytes/macrophages to enhance IL-23 secretion. Therefore, we predict that the lactic acid that is secreted by many tumor cells is a proinflammatory mediator that promotes tumor development.

Materials and Methods

**Cell culture**

The CADO-LC10 cell line, which was established from a human lung adenocarcinoma (22), was cultured in high glucose DMEM (4.5 mg/ml glucose; Sigma-Aldrich). Human PBMC, human monocytes, mouse splenocytes, the mouse macrophage-like cell line J774.1 (RIKEN cell bank), and the mouse melanoma cell line B16 were cultured in RPMI 1640 (Sigma-Aldrich). All media were supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C under a 5% CO₂ atmosphere.

**Reagents and Abs**

We purchased the TLR ligands: PGN of *Staphylococcus aureus* and LPS from Santa Cruz Biotechnology. Santa Cruz Biotechnology. Human IL-12p40, and human IL-6 (BioSource International) ELISA kits, and 10% FCS. In separate experiments, we inhibited lactic acid production with sodium lactate (Wako Pure Chemical and sodium lactate was purchased from WAKO). We used an anti-mouse IL-23p19 Ab (G23-8; E Bioscience) to neutralize IL-23 activity and rat IgG1 Ab (eBRG1; eBioscience) as an isotype control; both Abs were used at a concentration of 10 μg/ml. We purchased recombinant GM-CSF from PeproTech and anti-GM-CSF receptor α-chain Ab (S-20) from Santa Cruz Biotechnology.

**Conditioned medium analysis**

Conditioned medium was prepared from CADO-LC10 cells that had been cultured for 3 days. The medium was passed through a 0.22-μm pore size filter (Millipore) and stored at −80°C. For some experiments, the conditioned medium was subjected to molecular size fractionation by using Microcon YM-10 centrifugal filter devices, which separates molecules at the nominal 10-kDa molecular mass cutoff (Millipore). The flow-through fraction was supplemented with 10% FCS and the retentate (nominal 10-kDa molecular mass) was supplemented with 10% FCS and the retentate (nominal 10-kDa molecular mass cutoff (Millipore). The flow-through fraction was diluted with serum-free culture medium to obtain the original volume. In other experiments, the conditioned medium was treated with 50 μg/ml proteinase K at 37°C for 1 h. To remove proteinase K, the medium was passed through a Microcon YM-10 and the flow-through fraction was used for further experimentation. The control media were subjected to the same treatments as the conditioned media.

To examine how lactic acid concentrations in the culture medium of CADO-LC10 affects the subsequent enhancing activity of the conditioned medium, we cultured confluent CADO-LC10 cells for 3 days in fresh glucose-free DMEM (Invitrogen) supplemented with 1 or 4.5 mg/ml glucose and 10% FCS. In separate experiments, we inhibited lactic acid production by culturing the CADO-LC10 cells in the presence or absence of 20 mM oxamic acid (Sigma-Aldrich) for 2 days. The pH of the conditioned media and the lactic acid-containing media neutralized with NaOH was neutralized with a pH meter (Beckman Coulter) at 37°C under a 5% CO₂ atmosphere. t-Lactic acid concentrations in the conditioned media were measured by using a Determiner LA Kit (Kyoramedics). In the enhancing activity analysis, the conditioned media of CADO-LC10 cells described above were added to cells with an equal volume of conditioned medium (i.e., 50% of the original volume). In other experiments, the conditioned medium was treated with 50 μg/ml proteinase K at 37°C for 1 h. To remove proteinase K, the medium was passed through a Microcon YM-10 and the flow-through fraction was used for further experimentation. The control media were subjected to the same treatments as the conditioned media.

**Measurement of cytokines**

Human PBMC were isolated from healthy volunteers by using Ficoll-Paque Plus (GE Healthcare Bio-Sciences). Human monocytes were purified from the PBMC by using the MACS system (Miltenyi Biotech) and monocyte isolation kit II (Miltenyi Biotech). To measure human IL-23 production, 1.5 × 10⁵ monocytes were cultured in 96-well tissue culture plates in the presence or absence of lactic acid for 24 h and then treated with 10 μg/ml PGN for 24 h. The IL-23, IL-12/23p40, and IL-6 levels in the culture supernatants were measured by using human IL-23 (Bender MedSystems), human IL-12p40, and human IL-6 (BioSource International) ELISA kits, respectively. To measure mouse IL-23 production, 1.0 × 10⁵ J744.1 cells were stimulated with lactic acid and 10 μg/ml PGN. The measurements of mouse IL-23 production were performed by using a mouse IL-23 ELISA kit (E Bioscience).

Mouse splenocytes isolated from an OVA-specific, MHC class II-restricted αβ TCR-transgenic mouse, OT-II (25), were cultured at 5 × 10⁶ cells/well in 96-well tissue culture plates with 0.2 μg/ml OVA(323-339) peptides (BioSynth International) in the presence or absence of TLR ligands and lactate acid. After 4 days of incubation, the cytokines in the culture supernatants were measured by using IL-17A (R&D Systems) and IFN-γ (BioSource International) ELISA kits. These experiments using animals were conducted according to our institutional guidelines.

**Real-time RT-PCR**

Total RNA was isolated from cells by using the SV96 Total RNA Isolation System (Promega) according to the manufacturer’s instructions, after which it was treated with RNase-free DNase I. cDNA was synthesized at 42°C for 50 min by using oligo(dT)₁₂₋₁₄ primers and SuperScript III reverse transcriptase in the presence of RNase inhibitor (Invitrogen). Diluted cDNA samples were mixed with a pair of primers derived from human IL-23p19 or β-actin cDNA sequences and PCR was performed by using SYBR Green PCR master mix (Applied Biosystems) and an Applied Biosystems 7500HT sequence detection system. The following PCR primers were designed: for human IL-23p19, forward primer, 5′-AGGTGTTGGAG ATGGCTGTTGACC-3′ and reverse primer, 5′-GTCTGGAGCTGACTGTC GTCGAGCTCCT-3′; for human β-actin, 5′-ATGATGAAACCACCGT CACAACTCCTGAATG-3′ and reverse primer, 5′-GAAACCGAAGA CTGAGCAGGAAATG-3′; and for human β-actin, forward primer, 5′-TCA CCCACACTGTCGCCATCTACGA3′ and reverse primer, 5′-CACCGG AACCCGGTCACATCCGAATG-3′. Copy numbers were calculated from the amount of cDNA cloned into the pGEM-T easy vector (Promega) and normalized to β-actin. Mouse IL-23p19 activity was measured by using the TaqMan PCR Core reagent kit, TaqMan probes, and primer sets of the TaqMan Gene Expression assay system (for IL-23p19, Mm00518984_m1 and for β-actin, Mm00607931_s1; Applied Biosystems). The relative expression of IL-23p19 was normalized to that of β-actin and calculated by using the ΔΔCt method (16, 26).

**Luciferase assay**

A luciferase reporter plasmid for monitoring IL-23p19 transcription was constructed as follows. A 6.7-kb fragment of the human IL-23p19 gene from the Sau1 site (→ 6654 bp) to the ATG initiation site that contained the 5′-flanking region and 5′-untranslated region of the gene was amplified by PCR using the RP11-348M3 clone (Research Genetics) as the template. The fragment was then subcloned between the SacI and Ncol sites of the pGL3 promoter vector (Promega) to generate the p19−5′ luc vector. We also constructed a reporter gene plasmid containing the 2.7-kb 5′-flanking region of the human IL-23p19 gene as follows. The p19−5′ luc plasmid was digested with SacI and Xmn1, treated with T4 DNA polymerase (Takara Bio), and self-ligated. We then inserted the XhoI and BamHI fragments of the PGK promoter-driven neomycin resistance gene from pGKEM7-neoW into the SacI and Ncol I sites of the plasmid to generate the p19−5′ 2.7k luc neo vector. The luciferase reporter plasmid for monitoring IL-23p19 transcription was combined with the PGK promoter-driven luc neo vector. The annealed oligonucleotides were annealed: 5′-TCGGAGAAATGGGCTATCCCGCTGGGACATTTCCGCAACCGC-3′ and 5′-GGTTTGGCAGAAGTCCCCACGCGGAAATGGCCATTTCC-3′ (the underlined sequences indicate canonical NF-κB binding sites). The annealed oligonucleotides were then subcloned together with a SacI/NcoI fragment of the minimal promoter sequence of the pNFκB-Luc vector (Stratagene) between XhoI and Ncol I sites of the pGL3 promoter vector containing the PGK promoter-driven neomycin resistance gene to generate the pGL3-2.7eB luc neo vector. J744.1 cells were then transfected with the p19-5′ 2.7k luc vector and pGKEM7-neoW, the p19-5′ 2.7k luc neo vector, or pGL3-2.8B luc neo vector by using FuGENE 6 (Roche) according to the manufacturer’s instructions. The transfected cells were selected with 200 μg/ml G418. The cells were seeded in 96-well tissue culture plates at 1 × 10⁴ cells/well and incubated with stimulants for 24 h as described above. After incubation, the cells were lysed with GloLysis buffer (Promega) and the luciferase activity was measured by using the Bright-Glo luciferase assay system (Promega) and a Mithras LB940 multimode reader (Berthold Technologies).

**Small interfering RNA (siRNA)**

We purchased SMARTpool siRNA reagents for the human LDHA gene from Dharmacon and the AllStars negative control siRNA rhodamine from Qiagen. To subclone J744.1 and CADO-LC10 cells that had been transfected with pXmpartialGEMsiRNA Transfection Reagent (Roche) according to the manufacturer’s instructions. This transfection procedure was repeated on the second day to increase the RNA interference efficiency. On the fourth day,
Tumor cell-conditioned medium enhances IL-23p19 expression in monocytes/macrophages

To determine whether tumor-secreted factors might modulate the TLR ligand-stimulated production of IL-23 by monocytes/macrophages, we first generated medium conditioned by the lung adenocarcinoma cell line CADO-LC10. Monocytes isolated from normal human PBMC were then stimulated with PGN in the presence (LC10) or absence (medium) of the conditioned medium. PGN induced IL-23p19 transcription in human monocytes/macrophages and the transcription was significantly increased by the preincubation of the conditioned medium (Fig. 1A, left). The conditioned medium alone did not induce IL-23p19 transcription in unstimulated monocytes/macrophages (Fig. 1A, left). Similar results were obtained for PBMC stimulated with BCG-CWS (Fig. 1A, middle).

We then size-fractionated the conditioned medium and the control medium into two fractions bearing the >10-kDa or <10-kDa molecules and performed the same experiment described above. The PGN-stimulated expression of IL-23p19 in the monocytes/macrophages was more strongly enhanced by the lower molecular mass fraction (5.9-fold) than by the higher molecular mass fraction (1.7-fold) (Fig. 1A, left). Thus, it appears that the tumor cells secrete a small molecule that augments TLR ligand-induced IL-23p19 expression in monocytes/macrophages.

We also examined the effect of the conditioned medium on the transcriptional expression of IL-12/23p40 in human PBMC. Although the unfractonated medium and the higher molecular mass 4.5 mg/ml glucose (Glc). The control media (Medium) were subjected to the same treatments as the conditioned media (LC10). These media were added at 50% to determine the enhancing activity. The data represent mean values ± SD (n = 3). **, p < 0.01.
fraction clearly enhanced the transcription of IL-12/23p40, the lower molecular mass fraction did not (Fig. 1A, right, and data not shown). The enhancement of IL-12/23p40 expression was significantly inhibited by the anti-GM-CSF receptor α-chain Ab, suggesting that GM-CSF in the conditioned medium mainly enhanced IL-12/23p40 expression (data not shown).

The conditioned medium also enhanced the PGN-induced secretion of IL-23 by the mouse macrophage-like cell line J774.1 (Fig. 1B).

The conditioned medium enhances IL-23p19 promoter activity
To examine the effect of the conditioned medium on IL-23p19 gene promoter activity, we performed a luciferase reporter assay using p19-5′ luc, which is a luciferase reporter plasmid containing the 6.7-kb 5’-flanking DNA region of the human IL-23p19 gene (Fig. 1C). We first established several stable J774.1 cell lines that contained the reporter plasmid p19-5′ luc (J774-p19-5′ luc cells). When these cell lines were stimulated with PGN, IL-23p19 promoter activity was increased (Fig. 1D, left). A further increase was observed when the cells were treated with PGN in the presence of the conditioned medium (Fig. 1D, left). Here again, the lower molecular mass fraction of the conditioned medium was proficient in stimulating IL-23p19 promoter activity (Fig. 1D, left). We also generated additional luciferase reporter cells (J774-p19-5′ 2.7k luc) from p19-5′ 2.7k luc that contained only the 2.7-kb 5’-flanking region of the human IL-23p19 gene (Fig. 1C). The reporter activity of this plasmid in J774.1 cells was increased by TLR stimuli (BCG-CWS and PGN) and this effect was further increased by the conditioned medium (data not shown).

Thus, the conditioned medium augments the stimulatory effect of TLR ligands on IL-23p19 promoter activity.

Characterization of the small molecule in the conditioned medium responsible for the increase in TLR-stimulated IL-23p19 promoter activity
To identify the small molecule in the conditioned medium, we subjected the conditioned medium to further molecular size fractionation (29–31). Indeed, the conditioned media of CADO-LC10 was found to contain high concentrations of glucose. The conditioned medium prepared in DMEM supplemented with low (1 mg/ml) or high (4.5 mg/ml) glucose concentration does indeed affect the subsequent enhancement of TLR ligand-stimulated gene expression induced by TLR ligands. J774-p19-5′ luc cells were stimulated with 10 μg/ml PGN in the presence of 0 (Medium), 5, 10, 15, or 20 mM lactic acid (left) or with 0, 2.5, 5, 10, or 30 μg/ml PGN in the presence of 15 mM lactic acid (right). B, J774-p19-5′ luc cells were stimulated with 10 μg/ml BCG-CWS (left), 100 ng/ml LPS (middle), or 100 ng/ml Pam3CSK4 (right) in the presence or absence of 15 mM lactic acid. C, J774-p19-5′ 2.7k luc cells were stimulated with 10 μg/ml PGN in the presence or absence of 15 mM lactic acid. D, J774-p19-5′ luc cells (left) and J774-2xK luc cells (right) were stimulated with 10 μg/ml PGN in the presence or absence of 15 mM lactic acid. After incubation for 4, 8, and 24 h, the cells were lysed and their luciferase activities were measured. The data represent mean values ± SD (n = 3).

cells cultured in 4.5 or 1 mg/ml glucose were found to contain 26.4 and 7.8 mM lactic acid, respectively (final concentrations of 13.2 and 3.9 mM in our assay). Therefore, we hypothesized that lactic acid may be the most likely candidate tumor cell-secreted factor that enhances TLR ligand-stimulated IL-23p19 transcription.

Lactic acid in the conditioned medium enhances the IL-23p19 gene expression induced by TLR ligands
To examine whether lactic acid indeed enhances the IL-23p19 gene expression induced by TLR ligands, J774-p19-5′ luc cells were stimulated with PGN in the presence of lactic acid. The luciferase activity in J774-p19-5′ luc cells was increased by lactic acid in a dose-dependent manner (Fig. 2A, left) and this enhancing effect was found to be dose-dependent (Fig. 2B). In contrast, the luciferase activity induced by PGN was not significantly enhanced by lactic acid. The luciferase activity induced by LPS was not significantly enhanced by lactic acid (Fig. 2C). Therefore, we next examined the effect of lactic acid on TLR ligand-induced human IL-23p19 promoter activity. A, J774-p19-5′ luc cells were stimulated with 10 μg/ml PGN in the presence of 0, 5, 10, 15, or 20 mM lactic acid (left) or with 0, 2.5, 5, 10, or 30 μg/ml PGN in the presence of 15 mM lactic acid (right). B, J774-p19-5′ luc cells were stimulated with 10 μg/ml BCG-CWS (left), 100 ng/ml LPS (middle), or 100 ng/ml Pam3CSK4 (right) in the presence or absence of 15 mM lactic acid. C, J774-p19-5′ 2.7k luc cells were stimulated with 10 μg/ml PGN in the presence or absence of 15 mM lactic acid. After incubation for 4, 8, and 24 h, the cells were lysed and their luciferase activities were measured. The data represent mean values ± SD (n = 3).
Effect was observed at all concentrations of PGN (Fig. 2A, right). However, lactic acid alone had no detectable effect in this assay (Fig. 2A, left). Lactic acid at 10 mM was as effective as the conditioned medium, which contained 27.3 ± 1.09 mM lactic acid (final concentration of 13.7 ± 0.55 mM in our assay; Fig. 2A, left). The enhancing activity of lactic acid was also observed with cells stimulated with not only PGN but also other TLR ligands, namely, BCG-CWS, LPS, and Pam3CSK4 (Fig. 2B).

We next examined the region in the IL-23p19 promoter that was responsive to lactic acid by using J774-p19-5′ 5′-flanking region of the human IL-23p19 gene (Fig. 2C). Searching of a TRANSFAC database (32) with the TFSEARCH program version 1.3 (http://www.rwcp.or.jp/papia/) revealed four predicted NF-κB binding sites in a 2.7-kb 5′-flanking region (Fig. 1B, arrowheads). However, when we constructed J774.1 cells transfected with a luciferase reporter construct carrying canonical NF-κB binding sites and tested their responses to TLR ligands in the presence or absence of lactic acid, we did not observe any enhancing activity (Fig. 2D).

To test whether the lactic acid secreted from the tumor cells is indeed responsible for augmenting the TLR ligand-induced IL-23p19 promoter activity, we inhibited the production of lactic acid from CADO-LC10 cells with LDHA-specific siRNA. The expression of LDHA mRNA (Fig. 3A, left) and protein (Fig. 3A, right) in LDHA siRNA-transfected cells was reduced to <10 and 50% of that of control siRNA-transfected cells, respectively. We examined enhancing activity using these conditioned media (Fig. 3B). Alternatively, we inhibited the LDH activity by adding oxamic acid, an inhibitor of LDH (Fig. 3C). Both treatments significantly reduced the concentration of lactic acid in the conditioned medium and this was matched with a decreased ability of the conditioned medium to enhance TLR ligand-stimulated IL-23p19 promoter activity (Fig. 3, B and C). Thus, the lactic acid secreted by the tumor cells was largely responsible for the enhancing effect of the conditioned medium.
Lactate anions are transported together with protons into cells by monocarboxylate transporters (MCTs) in a pH-dependent manner (33). Therefore, to examine whether the enhancing activity of lactic acid depends on the pH of the medium, we incubated J774-p19.5 luc cells with 15 mM lactic acid in the presence of NaOH, which altered the pH of the medium (Fig. 4A). The enhancing activity of lactic acid was decreased in a NaOH dose-dependent manner. Furthermore, sodium lactate did not show any enhancing activity (Fig. 4B). The intracellular pH of the cells decreased rapidly upon incubation with lactic acid (Fig. 4C, left), suggesting that protons were transported into the cells along with lactic acid. However, in contrast to lactic acid, hydrochloric acid, which also decreased the intracellular pH, had no enhancing effect (Fig. 4, C, right, and D). These results suggest that only the lactate anion in its transportable state, but not the neutralized lactate anion or proton, was responsible for the enhancing activity.

Lactic acid enhances secretion of proinflammatory cytokines by human monocytes/macrophages

The IL-23p19 subunit is covalently linked to the IL-12/23p40 subunit to form an IL-23 heterodimer. The heterodimer is secreted by human monocytes/macrophages/DCs stimulated with TLR ligands (5, 34). To investigate whether lactic acid enhances IL-23 secretion, human monocytes were stimulated with PGN in the presence of lactic acid for 24 h (Fig. 5A, left). The secretion of IL-23 was enhanced 1.8-fold by lactic acid. When GM-CSF was only present, IL-23 secretion was elevated 1.8-fold by lactic acid. When GM-CSF was only present, IL-23 secretion was enhanced 10.6-fold. Similarly, the PGN-induced secretion of IL-12/23p40 was slightly increased by lactic acid but synergistically enhanced by the further addition of GM-CSF (Fig. 5A, right). These results indicate that lactic acid and GM-CSF cooperate to stimulate TLR ligand-induced IL-23 and IL-12/23p40 production by human monocytes/macrophages. Furthermore, lactic acid also enhanced the secretion of another proinflammatory cytokine, IL-6, from human monocytes/macrophages (Fig. 5B).

We also observed that BCG-CWS increased IL-23p19 transcription in PBMC, and like the conditioned medium (Fig. 1A, middle), lactic acid significantly enhanced this response in a dose-dependent manner (Fig. 5C, left). Conversely, lactic acid did not increase IL-12/23p40 transcription (Fig. 5C, right), suggesting that lactic acid specifically acts on IL-23p19 transcription.
Lactic acid enhances the IL-23/IL-17 pathway

Because lactic acid further enhanced TLR-stimulated IL-23 production, we predicted that lactic acid should enhance IL-23 production from APCs stimulated with TLR ligand, leading to increased IL-17 production by IL-17-producing T cells. We then used OT-II transgenic mice, which have OVA_{A123-319} peptide-specific T cells (25). In the presence of the OVA peptide, we examined the effect of lactic acid on the IL-17 production of OT-II mouse splenocytes that had been stimulated with the TLR ligand (PGN or BCG-CWS) for 4 days. Lactic acid remarkably enhanced the secretion of IL-17 induced by PGN and BCG-CWS (Fig. 6A, left, □ and ■). Anti-IL-23p19 Ab significantly inhibited the lactic acid-induced enhancement of IL-17 production (Fig. 6A, left, □ and ■). This result indicates that lactic acid indeed stimulates APCs to produce IL-23, which then drives peptide-activated T cells to produce IL-17. Interestingly, in the presence of the peptide, lactic acid induced IL-17 production even without the TLR ligand and this effect was inhibited by the anti-IL-23p19 Ab (Fig. 6A, left, and B). The peptide or lactic acid alone rarely induced IL-17 production by splenocytes (Fig. 6B).

We also examined the effect of lactic acid on the secretion of IFN-γ (Fig. 6A, right). When the TLR ligand was present along with the peptide, the splenocytes produced IFN-γ. However, lactic acid did not potentiate IFN-γ production under these conditions (Fig. 6A, right, □ and ■). In contrast to IL-17 production, lactic acid did not induce IFN-γ production when the TLR ligand was absent (Fig. 6A, right). Notably, the effect of lactic acid on IFN-γ production was different when the TLR ligand was replaced by LPS. Although lactic acid potentiated IL-17 production by splenocytes irrespective of the ligand used for stimulation (Fig. 6C, left), it severely inhibited IFN-γ production (Fig. 6C, right).

Lactic acid enhances the expression of IL-23p19 mRNA in tumor-infiltrating immune cells

Many immune cells infiltrate the tumor microenvironment and induce local inflammation in and around the tumor. We examined whether tumor-infiltrating immune cells actually have the ability to express a high amount of IL-23 in response to lactic acid. We purified CD45-positive cells (CD11b- and/or CD11c-positive cells, 64%) that had infiltrated the tumors formed by B16 melanoma cells (Fig. 7A). These cells were stimulated with BCG-CWS and lactic acid for 4 h. The level of IL-23p19 transcripts significantly increased in the presence of lactic acid (Fig. 7B). This result suggests that lactic acid also enhances the production of IL-23 in tumor-infiltrating immune cells, probably in macrophages and/or DCs.

Discussion

Tumor-secreted lactic acid enhances TLR ligand-dependent IL-23 expression in monocytes/macrophages

In this study, we sought to elucidate the mechanisms that induce immune cells in the tumor microenvironment to produce proinflammatory cytokines such as IL-23. We also asked, how does the tumor induce chronic inflammation without being attacked by the immune cells? We found that many tumor cell lines secrete factors that enhance TLR ligand-stimulated IL-23p19 transcription and IL-23 secretion by human and mouse monocytes/macrophages (Fig. 1A and data not shown). We clarified that the main factor responsible for this effect is a molecule smaller than 500 Da that is protease resistant, heat stable, and only secreted from tumor cell lines when they are cultured in high glucose medium (Fig. 1, D and E, and data not shown). We then discovered that this molecule is lactic acid (Figs. 2–5). Lactic acid increased the production of proinflammatory cytokines, IL-23, and IL-6 from human monocytes (Fig. 5).

GM-CSF also enhanced TLR ligand-induced IL-23 and IL-12/23p40 production (Fig. 5). GM-CSF is secreted by many tumor cells as well as CADO-LC10 cells (35) and increases the expression of TLR2 (36). We suggest that tumor-secreted GM-CSF and lactic acid cooperate to increase IL-23 production following TLR2 ligand stimulation.

Predicted lactic acid signaling pathway

Although lactic acid is completely ionized, even under neutralized conditions (37), the neutralization of lactic acid suppressed its ability to enhance IL-23p19 promoter activity in a pH-dependent manner (Fig. 4A). Moreover, neither sodium lactate nor acidification with hydrochloric acid enhanced IL-23p19 transcription (Fig. 4, B and D). Lactate anions are transported together with protons through MCTs and a pH gradient is necessary for effective transport of lactic acid into cells (33). Therefore, we predict that lactic acid transported into monocytes/macrophages by MCTs may be recognized by an intracellular sensor that, along with the TLR signal, activates the IL-23p19 promoter.

We found that DNA elements responsible for the enhancing activity of lactic acid are localized in the 2.7-kb 5’-flanking region of the human IL-23p19 gene (Fig. 2C). We found four predicted NF-κB binding sites in this region (Fig. 1C). Although NF-κB signaling plays an important role in the TLR signaling pathway (4), lactic acid did not enhance the luciferase activity of a reporter vector carrying canonical NF-κB-binding elements (Fig. 2D). Therefore, the lactic acid signal pathway may be independent of the NF-κB pathway.

Lactic acid is not only a terminal metabolite of glycolysis but also a proinflammatory mediator

In most normal mammalian cells, the metabolism from glucose to lactate is inhibited by the presence of oxygen, which leads to the oxidation of pyruvate to CO2 and H2O in the mitochondria. However, in cancer cells, glycolysis is up-regulated, even in aerobic conditions, a phenomenon known as the “Warburg effect” or “aerobic glycolysis” (29, 30). This results in the production of large amounts of lactic acid and the accumulation of lactic acid in the microenvironment of many cancer cell types (31). High concentrations of lactate in solid tumors such as cervical carcinoma

FIGURE 7. Lactic acid enhances the expression of IL-23p19 mRNA in tumor-infiltrating immune cells. A, Tumor-infiltrating CD45-positive cells were purified from tumors formed by B16 mouse melanoma cells and stained for CD11b and CD11c. The stained cells were analyzed by FACS. B, One hundred thousand tumor-infiltrating immune cells (CD45-positive cells, 86%) were stimulated with 10 μg/ml BCG-CWS in the presence or absence of 15 mM lactic acid. After 4 h, the expression of IL-23p19 and β-actin transcripts was measured by TaqMan RT-PCR. Relative expression was normalized to that of β-actin. The data represent mean values ± SD (n = 3). **, p < 0.01.

Lactic acid did not potentiate IFN-γ expression (Fig. 7 right).
(10.0 ± 2.9 μmol/g or >8.3 μmol/g) and head and neck cancer (>7.1 μmol/g) are associated with higher frequencies of distant metastasis (31, 38–40). Moreover, low lactate tumors in primary lesions are associated with longer disease-free survival than high lactate tumors. In addition, when the LDHA activity that induces the metabolism of pyruvate to lactate is suppressed, the tumorigenicity is severely diminished (41).

In this study, we showed that lactic acid enhances the expression of IL-23p19 in tumor-infiltrating immune cells activated by TLR stimuli (Fig. 7) and in splenocytes in the presence of Ag stimulus (data not shown) and induces the Ag- and IL-23-dependent secretion of IL-17 from splenocytes (Fig. 6). Significantly, we detected this IL-23-dependent enhancing activity even when TLR ligands were absent. Since lactic acid alone did not induce IL-23 production in the absence of Ag stimulation, we predicted that an interaction between APCs and T cells mediated by Ag and lactic acid causes IL-23-dependent IL-17 production in the absence of the TLR ligand. We observed that lactic acid severely inhibited IFN-γ production by Ag-stimulated splenocytes treated with LPS (Fig. 6C, right). The Kreutz group (42) reported that lactic acid represses the secretion of IL-12p70 by LPS-stimulated DCs. Therefore, lactic acid may inhibit IFN-γ production by suppressing the IL-12p70 production of monocytes/macrophages. In contrast, lactic acid did not affect IFN-γ production of Ag-stimulated splenocytes treated with PGN or BCG-CWS (Fig. 6A, right), it is assumed that PGN and BCG-CWS do not stimulate IL-12p70 production by monocytes/macrophages (14). These results suggest that upon antigenic stimulation of T cells, lactic acid mediates the activation of the IL-23/IL-17 pathway rather than the induction of IFN-γ-producing Th1 cells. We predict that lactic acid derived from tumor cells may induce inflammation but not the infiltration of CTLs in the tumor microenvironment, even in the absence of TLR ligand stimuli from microbial infections, and that inflammation promotes angiogenesis and tumor development.

The Kreutz group (42, 43) also reported that lactic acid downregulates the cytokine production and proliferation of CTLs. Furthermore, the Murray group (44) showed that MCT1 inhibitors, which inhibit the transport of lactic acid, suppress T lymphocyte proliferation. These groups suggested that a high concentration of extracellular lactic acid or inhibition of lactic acid excretion might cause intracellular accumulation of lactic acid with consequent disturbance of T cell metabolism and function. In contrast, our results indicate that lactic acid up-regulates IL-23p19 transcription in monocytes/macrophages.

In conclusion, our results show that lactic acid acts as a novel tumor-derived factor that strongly induces the IL-23/IL-17 proinflammatory pathway without inducing Th1 responses. Thus, the production and excretion of lactic acid appears to be not only essential for the proliferation of tumor cells with up-regulated glycolysis, but also for the induction of inflammation in the tumor microenvironment, which promotes tumor progression. Therefore, lactic acid and the lactic acid/IL-23 signal pathway may be an attractive target for treating tumors.

Acknowledgments

We thank K. Kawata, T. Yasuda, and C. Kozai in our laboratory for technical assistance and Dr. M. Saio (Gifu University, Gifu, Japan) for technical support. We also thank Drs. K. Toyoshima (RIKEN, Yokohama, Japan) and I. Azuma (Muroran Institute of Technology, Muroran, Japan) for providing BCG-CWS, Dr. W. R. Heath (The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) for providing OT-II mice, and Drs. J. Takeda and K. Yusa (Osaka University, Suita, Japan) for providing pGKEm7-neoW.

Disclosures

The authors have no financial conflict of interest.

References


