IL-20 Is Regulated by Hypoxia-Inducible Factor and Up-Regulated after Experimental Ischemic Stroke

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IL-20, an IL-10 family member, is involved in various inflammatory diseases, such as psoriasis, rheumatoid arthritis, and atherosclerosis. We investigated whether hypoxia in vitro and an in vivo model of ischemic stroke would up-regulate IL-20 expression. In vitro, IL-20 expression increased in hypoxic HaCaT, HEK293 cells, chondrocytes, monocytes, and glioblastoma cells. Inhibition of hypoxia-inducible factor 1α inhibited $CoCl_2$-induced IL-20 expression. We identified two putative hypoxia response elements in the human *il20* gene promoter. Promoter activity assays showed that $CoCl_2$ mimicked hypoxia-activated luciferase reporter gene expression. In vivo, experimental ischemic stroke up-regulated IL-20 in the sera and brain tissue of rats. IL-20 stained positively in glia-like cells in peri-infarcted lesions, but not in contralateral tissue. Administration of IL-20 mAb ameliorated ischemia-induced brain infarction of rats after experimental ischemic stroke. In vitro, RT-PCR analysis showed that glioblastoma cells, GBM8901, expressed IL-20 and its receptor subunits IL-20R1, IL-20R2, and IL-22R1. IL-20 induced cell proliferation in GBM8901 cells by activating the JAK2/STAT3 and ERK1/2 pathways. IL-20 also induced production of IL-1β, IL-8, and MCP-1 in GBM8901 cells. We conclude that IL-20 was responsive to hypoxia in vitro and in the ischemic stroke model and that up-regulation of IL-20 in the ischemic brain may contribute to brain injury. ***The Journal of Immunology,*** **2009, 182: 5003–5012.**

Stroke is the second leading cause of death and the leading cause of adult disability worldwide. Although different mechanisms are involved in the pathogenesis of stroke, increasing evidence shows that ischemic injury and inflammation account for its pathogenic progression (1, 2). Ischemic brain injury after stroke is a dynamic process that evolves over a period of hours to several days, particularly in the area surrounding the core of the infarct known as the penumbra (3). This process includes oxidative stress, cell death, and inflammation, as well as the activation of endogenous adaptive and regenerative mechanisms. The regulation of many of these processes occurs at the transcriptional level and involves the concerted activation of various transcription factors, including hypoxia-inducible factor 1α (HIF-1α)[2] (4).

Ischemic brain injury is a consequence of a severe reduction in the blood supply to the affected region. The deficits can often be permanent because adult neurons fail to regenerate. After they have been activated by injury, astrocytes and microglia release factors that recruit other astrocytes and microglia to the injury site. This process can lead to glial scar formation, which has the potential to block the growth and maturation of neural progenitors and to impede neovascularization, thus inhibiting recovery after injury (5).

Cytokines are up-regulated in the brain in a variety of diseases, including stroke, and are expressed not only in the cells of the immune system, but are also produced by resident brain cells, including glia cells and neurons (6–8). Chemokine expression precedes inflammatory cell infiltration following cerebral ischemia (9). IL-1β (10, 11), TNF-α (12, 13), IL-8 (14), and MCP-1 (15) appear to exacerbate cerebral injury; however, TGF-β (16) and IL-10 (17) may be neuroprotective.

The pleiotropic inflammatory cytokine IL-20, a member of IL-10 family which includes IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26 (18, 19), is expressed in monocytes, epithelial cells, and endothelial cells and exerts its biological functions on multiple cell types by activating IL-20R1/IL-20R2 or IL-22R1/IL-20R2 complexes (20). IL-20 is involved in various inflammatory diseases (21), such as psoriasis (18, 22, 23), rheumatoid arthritis (24), atherosclerosis (25, 26), and renal failure (27). Recently, IL-20 has been reported to regulate angiogenesis (28, 29). It is also an arteriogenic cytokine based on its actions in remodeling collateral networks and improving the functions of ischemic hind limbs (30).

Our previous study showed that hypoxia induced IL-20 in endothelial cells (26). Little is known about the molecular mechanism of gene regulation of IL-20 in hypoxia and its clinical implications. In the present study, we found up-regulation of IL-20 under hypoxic conditions in vitro and in the ischemic brain in vivo. We identified IL-20 promoter regions and the functional response elements of the *il20* gene in response to hypoxia. We also demonstrated a pathogenic role of IL-20 in ischemic brain injury in vivo using an animal model of transient middle cerebral artery occlusion (MCAO).

Materials and Methods

Cell culture, reagents, and hypoxia treatment

HaCaT and HEK293 cells were purchased from American Type Culture Collection and cultured in DMEM (Invitrogen) containing 10% FBS.

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Received for publication November 5, 2008. Accepted for publication February 10, 2009.

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[2] Abbreviations used in this paper: HIF-1α, hypoxia-inducible factor 1α; MCAO, middle cerebral artery occlusion; HRE, hypoxia response element; β-gal, β-galactosidase; 2ME2, 2-methoxy estradiol; MCA, middle cerebral artery; TTC, 2,3,5-triphenyltetrazolium chloride; GFAP, glial fibrillary acidic protein; TSS, transcriptional start site; MIF, migration inhibitory factor; 5′ RACE, rapid amplification of 5′ complementary DNA ends.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803653

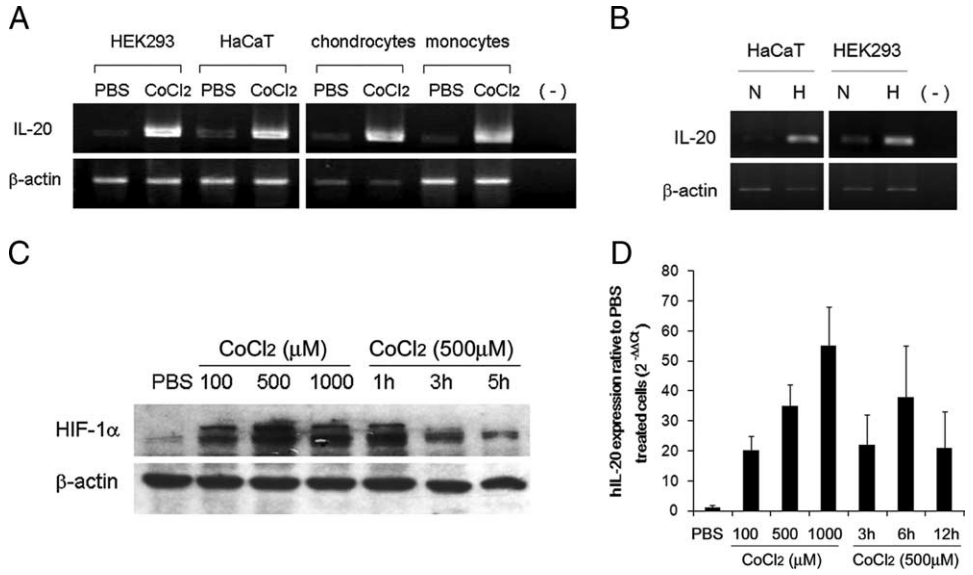

FIGURE 1. Regulation of IL-20 by hypoxia. *A*, HEK293, HaCaT, primary chondrocytes, and primary human monocytes were treated with PBS or $CoCl_2$ (100 μM) for 6 h and then RNA was extracted for RT-PCR analysis. *B*, Normoxic (N; 21% O_2) and hypoxic (H; 1% O_2) HaCaT and HEK293 cells were incubated for 24 h and then, using RT-PCR, IL-20 expression was analyzed. *C*, HEK293 cells were treated with $CoCl_2$ for 1 h at different dosages (100, 500, and 1000 μM) or with 500 μM $CoCl_2$ for different times (1, 3, and 5 h). Cell lysates were then analyzed using immunoblotting with anti-human HIF-1α mAb. *D*, HEK293 cells were treated with $CoCl_2$ for 1 h at different dosages (100, 500, and 1000 μM) or with 500 μM $CoCl_2$ for different times (3, 6, and 12 h). RNA was then analyzed using real-time PCR with IL-20-specific primers. The relative quantification of PCR products was expressed as $2^{-\Delta\Delta Ct}$, corrected using HPRT expression, and relative to levels of PBS-treated cells. Representative results were obtained from three independent experiments.

Primary human chondrocytes were isolated and maintained in DMEM/F12 medium (Invitrogen) containing 10% FBS. Human peripheral monocytes were freshly prepared before experiments as previously described (26). Human GBM8901, a glioblastoma cell line, was purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan) and was maintained in DMEM (Invitrogen) containing 10% FBS.

For $CoCl_2$ (Sigma-Aldrich) treatment to mimic hypoxia or incubation in a hypoxia (1% O_2) chamber, cells were treated and cultured in serum-free medium for RT-PCR and ELISA analyses. To analyze the cytokine production in GBM8901, cells were cultured in serum-free medium and treated with IL-20 (R&D Systems) or IL-1β (PeproTech) for 24 h. After treatment, the conditioned medium was collected and analyzed using specific ELISA kits.

Plasmids, transfection, and promoter activity assays

A full-length human IL-20 cDNA clone was obtained by repetitive rapid amplification of 5′ complementary DNA ends (5′ RACE) using anchor

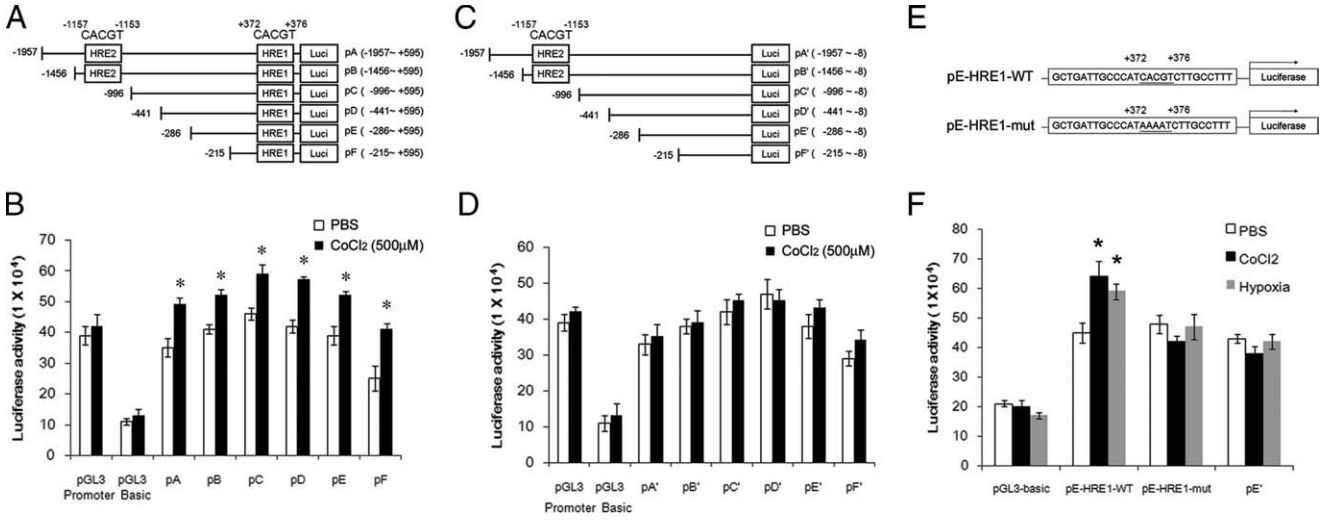

FIGURE 2. Promoter activity of the human IL-20 gene. *A*, A schematic drawing of the promoter constructs of IL-20 (pA, pB, pC, pD, pE, and pF) containing HRE1 and HRE2. *B*, The promoter constructs were transfected into HEK293 cells for 8 h and treated with PBS or $CoCl_2$ (500 μM) for 24 h. Cell lysates were then analyzed using a luciferase assay. *C*, A schematic drawing of the promoter constructs of IL-20 without HRE1. *D*, The promoter constructs (pA′, pB′, pC′, pD′, pE′, and pF′) were transfected into HEK293 cells for 8 h and treated with PBS or $CoCl_2$ (500 μM) for 24 h. Cell lysates were analyzed using a luciferase assay. $*$, $p < 0.05$, compared with the PBS-treated group. Representative results were obtained from three independent experiments. *E*, A schematic drawing of the promoter constructs of pE with HRE1 mutation (CACGT to AAAAT). *F*, The promoter constructs (pGL3-basic, pE-HRE1-WT, pE-HRE1-mut, and pE′) were transfected into HEK293 cells for 8 h and treated with PBS or $CoCl_2$ (500 μM) or hypoxia (1% O_2) for 24 h. $*$, $p < 0.05$, compared with the PBS-treated group.

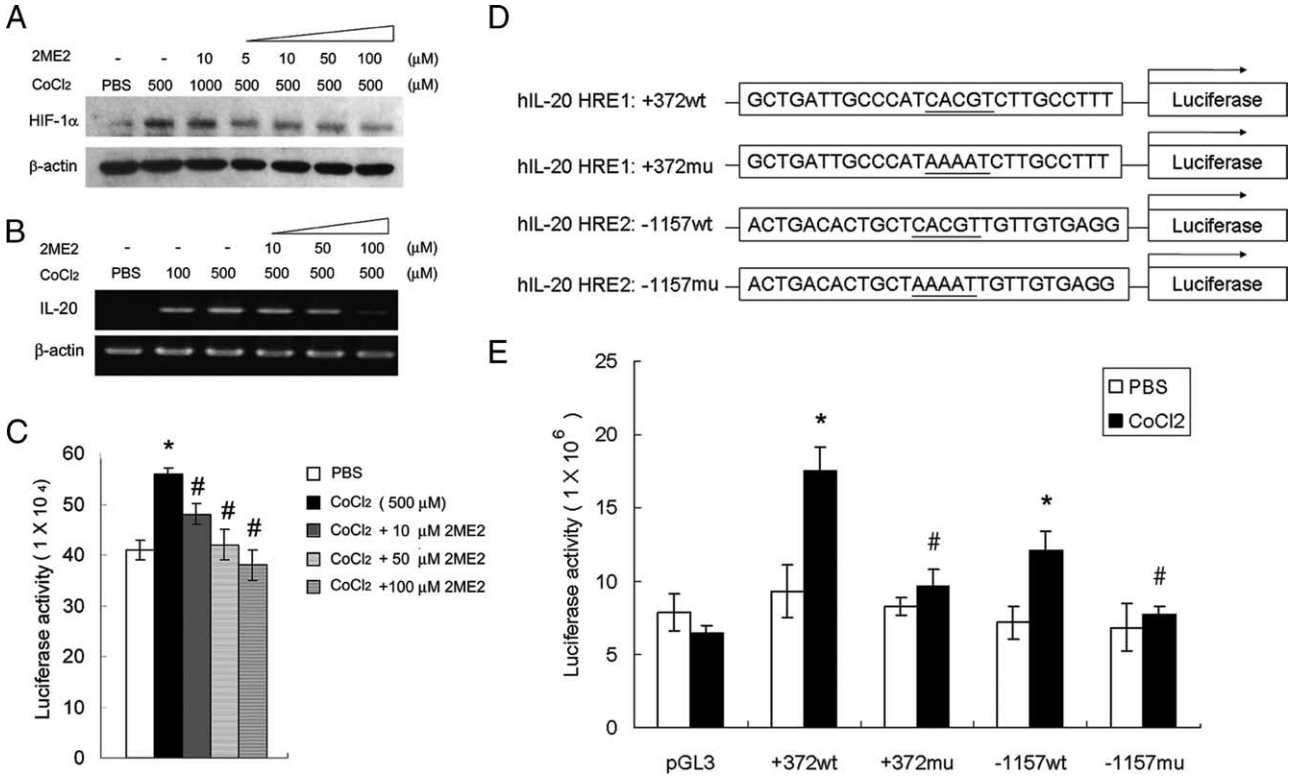

FIGURE 3. Identifying two HREs in the IL-20 promoter. Immunoblotting of HIF-1α (*A*) and RT-PCR analysis (*B*) of IL-20 in HEK293 cells treated with $CoCl_2$ with different dosages of 2ME2. *C*, The effect of 2ME2 on IL-20 promoter activity. HEK293 cells were transfected with the IL-20 promoter construct pE and incubated with $CoCl_2$ (500 μM) and 2ME2 (10, 50, and 100 μM). *, $p < 0.05$, compared with the PBS-treated group. #, $p < 0.05$, compared with the $CoCl_2$-treated group without 2ME2. Representative results were obtained from three independent experiments. *D*, A schematic drawing of the construction of wild-type (wt) and mutant (mu) HRE1 and HRE2 in the human IL-20 in the pGL3 promoter vector. The 27-bp HRE-binding matrices of the human IL-20 gene were inserted between the *Kpn*I and *Nhe*I sites of the pGL3 promoter vectors. In the mutant constructs, the conserved HRE sequence (underlined) was changed to AAAAT. *E*, HEK293 cells transfected with the constructs were treated with $CoCl_2$ (500 μM) and analyzed using a luciferase assay. *, $p < 0.05$, compared with the PBS-treated group. #, $p < 0.05$, compared with the group transfected with wild-type HRE1 and HRE2. Representative results were obtained from three independent experiments.

primers and gene-specific antisense primers: 5′-gtggaaggagtccataggag-3′ (first PCR) and 5′-aggagctaggaattcaaagaag-3′ (second PCR). After three rounds of 5′ RACE, the 5′ end of exon 1 (transcriptional start site) was determined and designated as nucleotide 1 (supplemental Fig. 1[3]).

We used PCR with specific primers to amplify 12 different regions upstream of exon 1 of the human *il20* gene from the DNA of the BAC clone (RP11-564A8). Twelve fragments (pA, pB, pC, pD, pE, pF, pA′, pB′, pC′, pD′, pE′, and pF′) containing different lengths of sequences were inserted between the *Kpn*I and *Nhe*I sites of the pGL3-basic vector (Promega) containing the entire coding sequences of firefly luciferase: pA (−1957/−8), pB (−1456/−8), pC (−966/−8), pD (−441/−8), pE (−286/−8), pF (−215/−8), pA′ (−1957/+595), pB′ (−1456/+595), pC′ (−966/+595), pD′ (−441/+595), pE′ (−286/+595), and pF′ (−215/+595). Alternatively, oligonucleotides corresponding to the putative hypoxia response element (HRE)-binding matrixes (27 bases) of the human *il20* gene promoters, HRE1 (+372/+376) and HRE2 (−1157/−1153), were cloned into SV40-driven pGL3 promoter vector after annealing. The putative HRE sequence was mutated by replacing the base CACGT with AAAAT.

HEK293 cells or GBM8901 cells (1×10^6/well) were seeded in 6-well plates for luciferase assays. Cells were transfected with 1 μg of plasmid DNA from the fusion gene and 0.4 μg of the β-galactosidase (β-gal) gene, which was used as an internal transfection efficiency control by using 1 μl of a reagent (Lipofectamine 2000; Invitrogen). Twenty-four hours after transfection, the medium was replaced with fresh medium and the cells were subjected to $CoCl_2$ (500 μM)-mimicked hypoxic treatment for another 24 h. Transfected cells were then collected for an analysis of luciferase activity. In addition, to test the effect of HIF-1α on the *il20* promoter, we transfected cells with construct pE and treated cells with $CoCl_2$ (500 μM) with the HIF-α inhibitor 2-methoxyestradiol (31) (2ME2; Sigma-Aldrich) for 24 h and analyzed the luciferase activity.

Cells were collected and the luciferase activity was analyzed according to the manufacturer's protocol (Promega). To obtain internal control of β-gal gene transfection, the cell lysate was also used for β-gal activity analysis. The luciferase activity from each promoter-fusion gene was divided by β-gal activity to obtain a true representation of luciferase activity from each promoter-luciferase fusion gene. Each luciferase assay experiment was performed in triplicate and repeated as indicated in the figure legends.

The transfection efficiency was tested by transfection of the cells with pEGFP-N1. The GFP-positive cells were ~90% in HEK293 cells and ~60% in GBM8901 cells after a 24-h transfection with the pEGFP-N1 vector.

RT-PCR

Total RNA from $CoCl_2$-, hypoxia-, or hIL-20-treated cells was extracted using TRIzol (Invitrogen) and then total RNA underwent reverse transcription using SuperScript II (Invitrogen) according to the manufacturer's instructions. Different genes were amplified using PCR with specific primers. PCR products were visualized on 2% agarose gels containing ethidium bromide. Primer sequences used for PCR are listed in supplemental Table I.

Real-time quantitative PCR

Detection of amplified template was accomplished with SYBR Green I (Bio-Rad) chemistry using an MJ Research DNA Engine Opticon 2 fluorescence detection system. Individual PCR contained 10 μl of cDNA (diluted 1/50), 1.25 μl of 10 μM forward primers, 1.25 μl of 10 μM reverse primers, and 12.5 μl of SYBR Green I in a final volume of 25 μl. Individual PCR products were analyzed using melting point analysis. Samples were heated from 50 to 95°C, and the decline in fluorescent signals of each

[3] The online version of this article contains supplemental material.

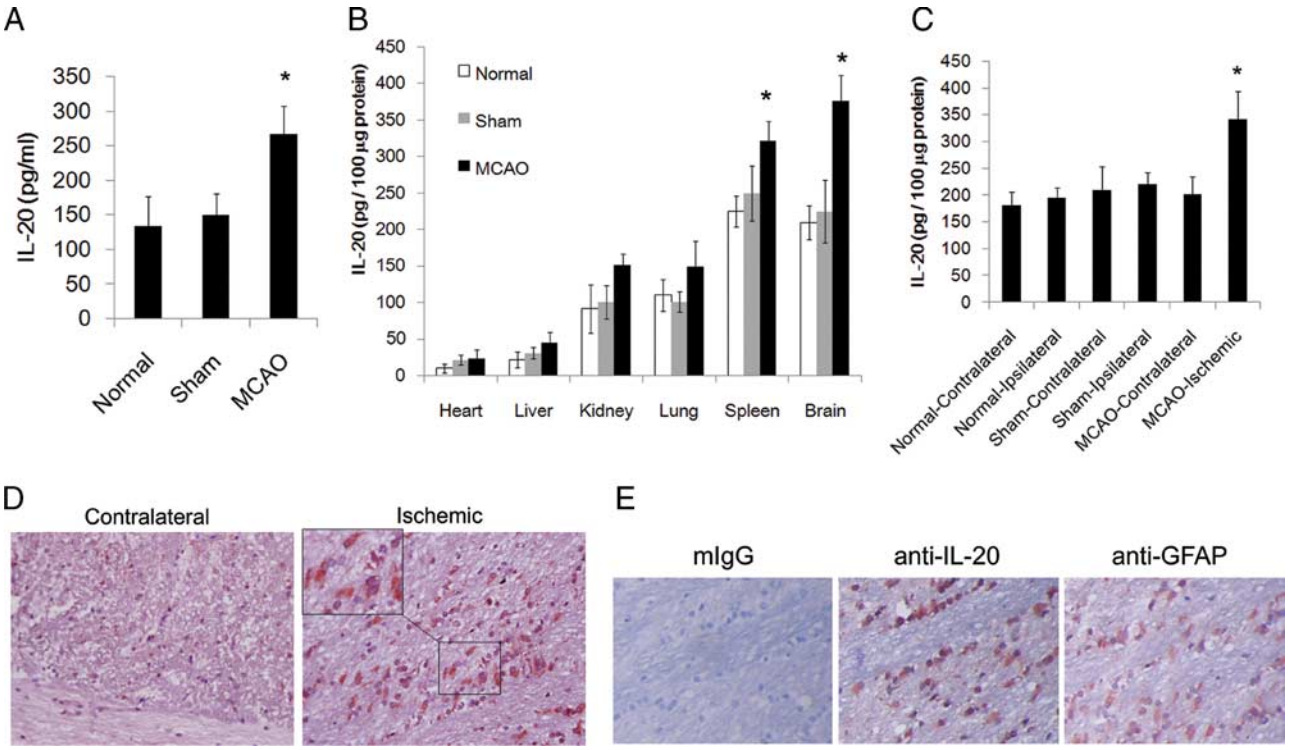

FIGURE 4. Up-regulation of IL-20 in the MCAO-induced ischemic stroke. *A*, Serum was collected from normal, sham-operated controls, and MCAO-treated rats ($n = 10$ each) 3 days after MCAO and analyzed using ELISA. *B*, Tissues of heart, liver, kidney, lung, spleen, and brain from normal, sham-operated controls, and MCAO-treated rats were collected and homogenized ($n = 5$ in each group). The protein level of IL-20 in the tissue homogenates was analyzed using ELISA. *, $p < 0.05$, compared with sham controls. *C*, Specific regions of whole contralateral and ipsilateral hemispheres from brain tissue were collected from normal, sham-operated controls, and MCAO-treated rats ($n = 3$ each) 3 days after MCAO and the brain homogenates were analyzed using ELISA. *, $p < 0.05$, compared with the MCAO-contralateral group. *D*, Immunohistochemical staining of IL-20 in brain sections of contralateral (*left panel*) and ischemic ipsilateral (*right panel*) hemispheres collected from rats 3 days after MCAO. Square in *right panel* represents a high- power field of a selected region. *E*, The adjacent slides of the brain section from MCAO-treated rats after 3 days were stained with isotype mouse IgG (mIgG), anti-IL-20 (7E), and anti-GFAP. Photographs of brain slides are from representative experiments.

individual sample was assessed. The fluorescence/time-dependent generation of signals was assessed using the manufacturer's software program.

Animals and experimental procedures

Male Sprague Dawley rats (200–300 g) obtained from the Laboratory Animal Center (National Cheng Kung University, Tainan, Taiwan) were maintained on standard laboratory chow and drinking water (ad libitum). They were handled according to the guidelines given by the Council for the International Organization of Medical Sciences on Animal Experimentation (World Health Organization, Geneva, Switzerland) and the guidelines of the National Cheng Kung University. Transient focal cerebral ischemia was induced using intraluminal occlusion of the right middle cerebral artery (MCA). The root of the right MCA was occluded by inserting a silicone-coated 4.0 nylon thread from the bifurcation of the internal and external carotid arteries. The tip of the thread was placed 18 mm distal to the bifurcation. The thermocouple needle probe was inserted into the temporal muscle to maintain the temperature at 37.0 ± 0.1°C with a heating lamp during surgery. For ischemic reperfusion, the cerebral blood flow was restored 90 min after MCAO by pulling the thread by 5 mm. After the surgical incision was sutured, the rats were allowed to recover from anesthesia. After 72 h of MCAO treatment, rats were tested for neurological deficits and scored as previously described (32): 0, no observable neurological deficit (normal); 1, failure to extent contralateral forepaw (mild); 2, circling to the contralateral side (moderate); and 3, loss of walking or righting reflex (severe). Rats after MCAO treatment with severity scores from mild to severe (categories 1–3) were analyzed for IL-20 expression in serum, vital organs, and brain. For Ab treatment, anti-IL-20 mAb (10 mg/kg) was i.v. injected into rats immediately after ischemic reperfusion and injection of PBS was used as negative control.

Three days after MCAO, rats were sacrificed and the brains were collected for lesion analysis. The cerebral tissues were cut into seven equally spaced (2-mm) sections. The sections were incubated with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) at 37°C for 30 min with gentle shaking and then were fixed with 10% formalin in PBS. The stained slices were photographed and the size of the infarct was quantified using imaging software (Adobe Photoshop 10). The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated. Lesion volume was presented as a volume percentage of the lesion compared with the contralateral hemisphere.

Immunoblotting and ELISA

HIF-1α expression was analyzed using immunoblotting with anti-HIF-1α mAb (Sigma-Aldrich). Cell lysates from GBM8901 cells treated with IL-20 for 10 min were analyzed using immunoblotting with specific Abs against phospho-STAT3, phospho-ERK1/2, phospho-AKT, and phospho-JNK (Cell Signaling Technology). β-Actin was a loading control.

For analyses of the IL-20 level in the tissues, the vital organs, heart, kidney, lung, liver, spleen, and brain (ipsilateral hemisphere), were collected from normal healthy rats, sham-operated controls, and MCAO groups. Supernatants from homogenates were used to determine the expression of IL-20 with a commercially available IL-20 ELISA kit (PeproTech) according to the manufacturer's instructions. For analysis of the IL-20 level on the specific region of brain, the whole contralateral and ipsilateral hemispheres from normal healthy rat, sham-operated controls, and MCAO groups were separately dissected for homogenization. Supernatants from homogenates were similarly analyzed by ELISA.

GBM8901 cells were treated with PBS, IL-20 (100 ng/ml, 200 ng/ml), anti-IL-20 mAb (7E, 1 μg/ml), 7E (1 μg/ml) plus IL-20 (200 ng/ml), IL-1β (30 ng/ml), and IL-1β (30 ng/ml) plus IL-20 (200 ng/ml) for 24 h. Conditioned medium was collected and used to detect protein levels with

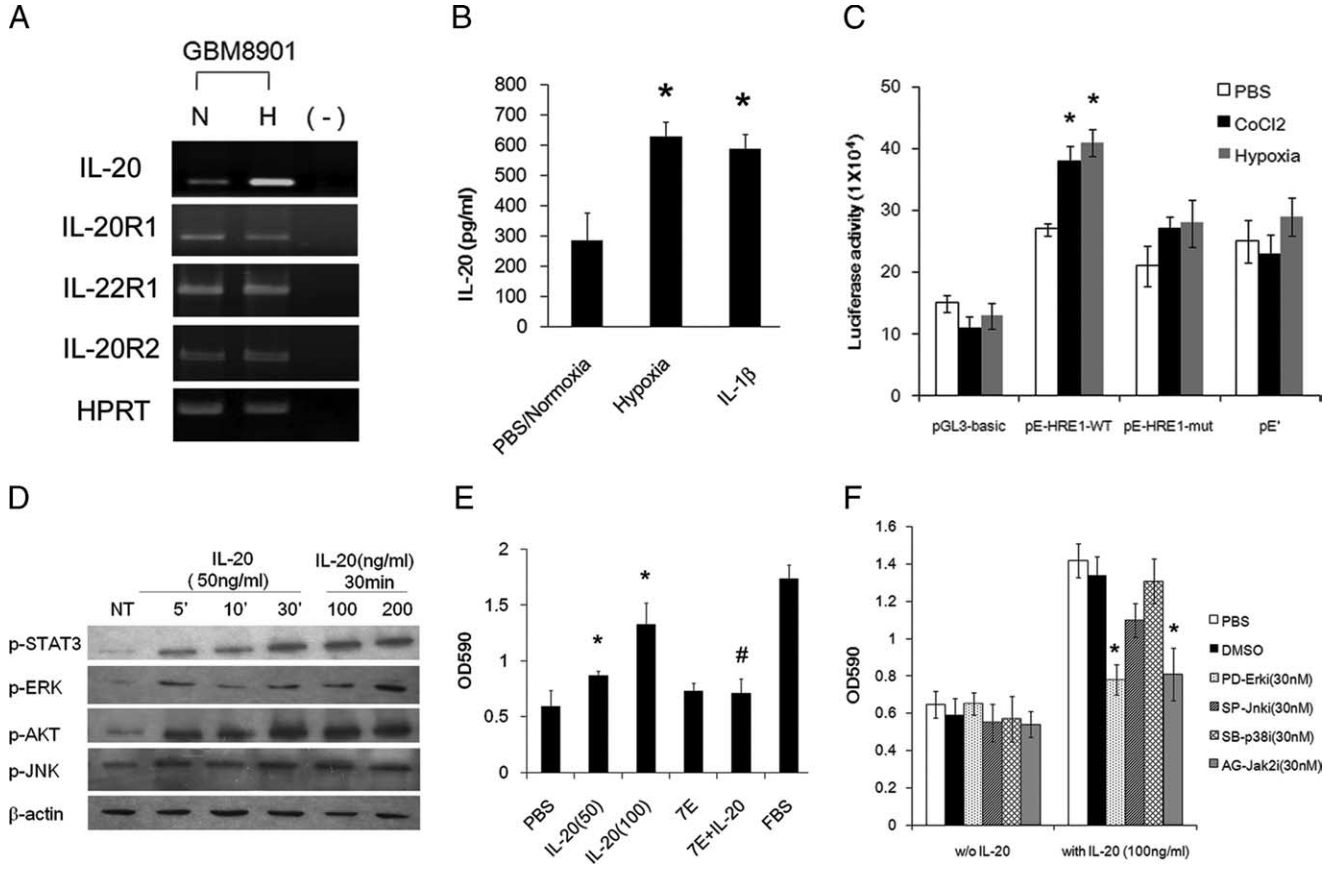

FIGURE 5. Functions of IL-20 in GBM8901 glioblastoma cells. *A*, RT-PCR analysis of the expression of IL-20 and its receptors in GBM8901 cells. *B*, ELISA analysis of IL-20 expression in the culture medium of normoxic (N; 21% O_2) and hypoxic (H; 1% O_2) GBM8901 cells incubated for 24 h. IL-1β (30 ng/ml) was treated as a positive control. *, $p < 0.05$, compared with normoxic cells. *C*, The promoter constructs (pGL3-basic, pE-HRE1-WT, pE-HRE1-mut, and pE') were transfected into GBM8901 cells for 8 h and treated with PBS or $CoCl_2$ (500 μM) or hypoxia (1% O_2) for 24 h. *, $p < 0.05$, compared with the PBS-treated group. *D*, Immunoblotting analyses of the phosphorylation of signal transduction molecules in GBM8901 cells after IL-20 treatment. *E*, An MTT assay analysis of the cell proliferation of GBM8901 cells in response to treatment with IL-20 (50 and 100 ng/ml), anti-IL-20 mAb (7E; 1 μg/ml), 7E (1 μg/ml) plus IL-20 (100 ng/ml), and 10% FBS (the positive control). *F*, An MTT assay analysis of the proliferation of GBM8901 cells treated with inhibitors of signal transduction molecules with or without (w/o) IL-20 for 72 h. *, $p < 0.05$, compared with the IL-20-treated group. Representative results were obtained from three independent experiments.

ELISA kits for IL-1β, IL-8, MCP-1, migration inhibitory factor (MIF), TNF-α, and IL-6 (R&D Systems).

Immunohistochemical staining

Paraffin-embedded rat brain sections were processed for immunohistochemistry staining using mAbs against IL-20 (7E) as previously described (23, 33). Isotype mouse IgG_1 was the negative control. Anti-Glial fibrillary acidic protein (GFAP) was used as a marker of astroglia (DakoCytomation). Immunoreactivity was detected using a 3-amino-9-ethylcarbazole substrate kit for peroxidase (DakoCytomation) and the nuclei were counterstained with Mayer's hematoxylin (ThermoShandon). For the quantification of IL-20-positive cells in the ischemic-infarcted lesion in the right MCA cortex and adjacent penumbra, six different fields at $\times$400 magnification were randomly chosen and the average IL-20-positive cells were calculated in each field. The result was obtained from the brain sections of five different rats in each group.

Cell proliferation assay

For cell proliferation, the MTT assay was performed as previously described (29). Cells incubated with the signal transduction inhibitors were treated with or without IL-20 for 72 h. The inhibitors (PD98059, ERK inhibitor; SP600125, JNK inhibitor; SB203580, p38 inhibitor; and AG490, JAK2 inhibitor) were purchased from Sigma-Aldrich.

Statistical analysis

Significant differences were evaluated using Student's *t* test with a statistical software package in Microsoft Excel. Statistical significance was set at $p < 0.05$.

Results

Induction of IL-20 by true and $CoCl_2$-mimicked hypoxia

Hypoxia induced HIF-1α activation and up-regulated angiogenic cytokines. To examine IL-20 mRNA expression during hypoxia, we treated four different types of cells with $CoCl_2$ to induce chemically mimicked hypoxia (34, 35). RT-PCR results showed that $CoCl_2$ up-regulated IL-20 mRNA levels in HaCaT (keratinocytes), HEK293 (human embryo kidney epithelial cells), primary chondrocytes, and primary monocytes (Fig. 1*A*). True hypoxia (1% O_2) also up-regulated the IL-20 mRNA level in HaCaT and HEK293 cells (Fig. 1*B*). To confirm that the $CoCl_2$-mimicked hypoxia reflected true hypoxia, we used immunoblotting to analyze HIF-1α protein levels. $CoCl_2$ dose-dependently increased HIF-1α levels, which peaked after 1 h in HEK293 cells (Fig. 1*C*). Real-time PCR showed that the IL-20 mRNA levels peaked after 6 h (Fig. 1*D*). These results suggested that IL-20 is a hypoxia-responsive gene and is up-regulated by hypoxia in different types of cells.

Promoter activity of il20 *gene promoter constructs*

In vitro assays showed that IL-20 was up-regulated by true and $CoCl_2$-mimicked hypoxia (Fig. 1). To determine whether hypoxia mediates the regulation of *il20* gene promoter activity, we first identified the transcriptional start site (TSS) of the human

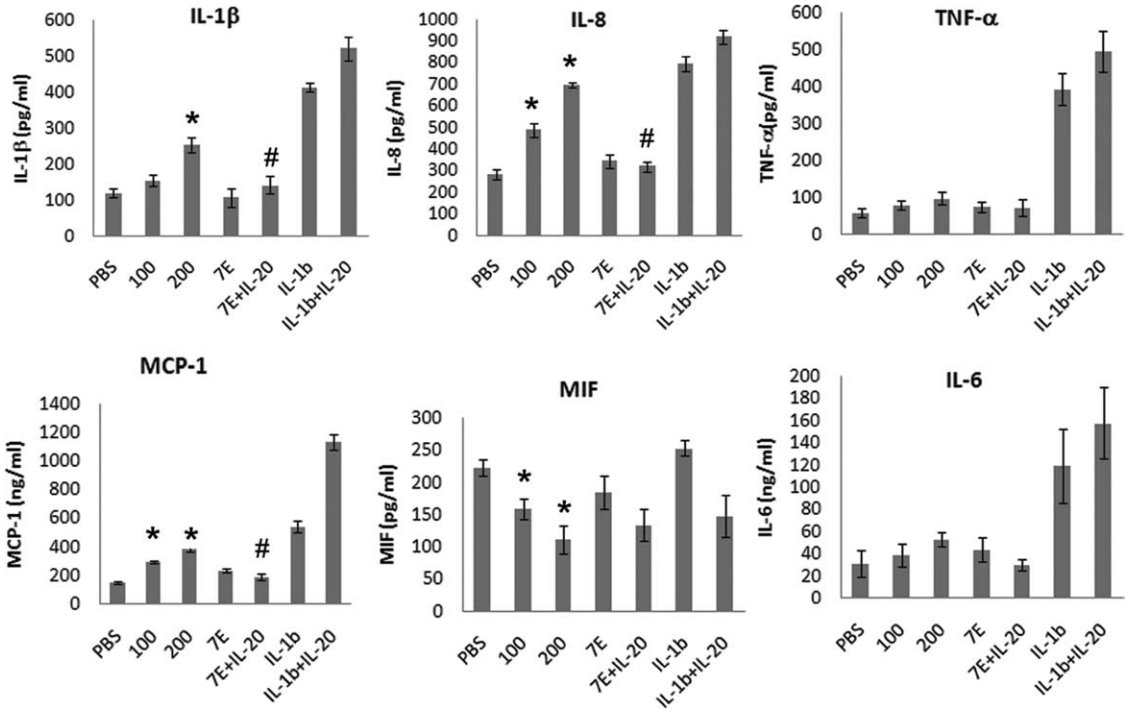

FIGURE 6. Induction of cytokines and chemokines in GBM8901 cells by IL-20. GBM8901 cells were treated with PBS, IL-20 (100 and 200 ng/ml), anti-IL-20 mAb (7E; 1 μg/ml), 7E (1 μg/ml) plus IL-20 (200 ng/ml), IL-1β (30 ng/ml), and IL-1β (30 ng/ml) plus IL-20 (200 ng/ml) for 24 h in serum-free conditions. The conditioned medium was collected and analyzed with ELISA kits for IL-1β, IL-8, MCP-1, MIF, TNF-α, and IL-6. *, $p < 0.05$, compared with the PBS-treated group. #, $p < 0.05$, compared with the IL-20 (200 μg/ml)-treated group. Representative results were obtained from three independent experiments.

il20 gene using a 5′ RACE series, and the TSS was designated "nucleotide 1." To characterize the DNA sequences involved in regulating human *il20* gene expression, we used PCR to amplify six potential promoter fragments (pA, pB, pC, pD, pE, and pF) using a human genomic clone (RP-11-564A8) as a template. All six promoter fragments contained at least one TATA box. The sizes of the PCR fragments ranged from −1957 to +595 according to the TSS (Fig. 2*A*). We also identified two putative HREs containing the conserved sequence (5′-CACGT-3′) in the *il20* gene promoter, HRE1 (+372/+376), and HRE2 (−1157/−1153). IL-20 was endogenously expressed in HEK293 cells and up-regulated by $CoCl_2$-mimicked hypoxia (Fig. 1). Thus, we used HEK293 cells for the analysis of promoter activity. After we had transfected the HEK293 cells with the promoter constructs, their luciferase activities were analyzed. All six constructs showed positive promoter activity, with the pC fusion gene the highest, 4- to 5-fold higher than the negative control of pGL3-basic vector. In response to $CoCl_2$-mimicked hypoxia, all six *il20* gene promoter constructs showed increased luciferase activity compared with the PBS-treated control (Fig. 2*B*). The construct containing only HRE1 (pC, pD, pE, and pF) preserved $CoCl_2$-increased promoter activity, which suggested that HRE1 is sufficient to mediate hypoxia-induced promoter activity. To confirm this result, we generated another six promoter constructs ranging from −1157 to −8 upstream of the TSS sites (pA′, pB′, pC′, pD′, pE′, and pF′) lacking HRE1 (Fig. 2*C*). Promoter activity assays revealed that deleting the region from −8 to +595 did not alter the original promoter activities, whereas the absence of HRE1 abolished the $CoCl_2$-increased promoter activity of the *il20* gene, suggesting that HRE1 was more important than HRE2 for hypoxia responsiveness (Fig. 2*D*).

To further confirm the specificity of HRE1 in the regulation of IL-20 expression in response to $CoCl_2$ or hypoxia treatment, we generated the mutant construct (pE-HRE1-mut) with mutation of the core HRE sequence (CACGT to AAAAT) and compared the promoter activity with the wild-type construct (pE-HRE1-WT) (Fig. 2*E*). Mutation of the core sequence of HRE1 abolished $CoCl_2$ or hypoxia-induced IL-20 promoter activity, indicating that HRE1 was specifically responsive to hypoxia. (Fig. 2*F*).

Inhibition of $CoCl_2$-induced IL-20 by 2ME2, an HIF-1α inhibitor

HIF-1α is the master transcription factor mediating the cellular response after hypoxia. To further investigate whether HIF-1α up-regulates IL-20 during hypoxia, we treated HEK293 cells with $CoCl_2$ and 2ME2. Western blotting showed a lower level of $CoCl_2$-induced HIF-1α protein in 2ME2-treated cells (Fig. 3*A*). RT-PCR showed that 2ME2 decreased the mRNA level of IL-20 transcripts (Fig. 3*B*). In the promoter activity assay, we transfected HEK293 cells with the IL-20 promoter construct pE and found that 2ME2 dose-dependently inhibited the $CoCl_2$-increased luciferase activity of the IL-20 promoter (Fig. 3*C*). These results indicated that HIF-1α up-regulated IL-20 during $CoCl_2$-mimicked hypoxia.

Identification of the functional HREs in il20 *gene promoter*

Two putative HREs containing the core sequence (5′-CACGT-3′) in the human *il20* gene promoter were identified as HRE1 (+372/+376) and HRE2 (−1157/−1153). HRE1 was essential for the $CoCl_2$-enhanced promoter activity of the *il20* gene. The 27 bases

of the HRE-binding matrix fragments of the human *il20* gene promoter were inserted between the *Kpn*I and *Nhe*I sites of the pGL3-promoter vector (Fig. 3*D*). HEK293 cells were transiently transfected with plasmids containing the 27 bases of HRE- binding matrices and were treated with $CoCl_2$ to test whether the HREs were functional. $CoCl_2$ significantly increased the promoter activity of the constructs containing HRE1 and HRE2 compared with those in PBS-treated control cells ($p < 0.05$). Promoter activity of HRE1 was higher than that of HRE2 under $CoCl_2$ treatment ($p < 0.05$). Furthermore, mutation of the core HRE sequence (CACGT to AAAAT) abolished $CoCl_2$-induced promoter activity of the wild-type HRE constructs (Fig. 3*E*). These results further confirmed that the two HREs of the *il20* gene are functional and that HRE1 was the primary hypoxia regulatory element.

Up-regulation of IL-20 in focal MCAO-induced ischemic stroke

To further investigate whether hypoxia in vivo up-regulates IL-20 expression and its significance in clinics, we performed a transient MCAO and reperfusion to induce experimental ischemic stroke. After 72 h of MCAO and reperfusion, the serum IL-20 level was significantly higher than in the normal and sham control group ($p < 0.05$; Fig. 4*A*). To further determine the source of IL-20 in serum after MCAO, we analyzed the protein levels of IL-20 in the heart, lung, liver, kidney, spleen, and brain from the rats that underwent 72 h of MCAO and reperfusion. IL-20 was significantly up-regulated in the spleen and brain in the MCAO group ($p < 0.05$; Fig. 4*B*). In addition, the protein level of IL-20 in the ischemic hemisphere was higher than in the contralateral hemisphere of the normal, sham control, and MCAO groups ($p < 0.05$; Fig. 4*C*). The contralateral expression of IL-20 in the MCAO group was not significantly different from those in both hemispheres of the sham control group, which indicated that IL-20 up-regulation was restricted to ischemic-infarcted lesions. Furthermore, immunohistochemical staining with IL-20 Ab showed that glia-like cells were positively stained in the ischemic-infarcted lesion in the right MCA cortex and adjacent penumbra. In contrast, contralateral brain tissue was only lightly stained (Fig. 4*D*). To prove that IL-20-positive cells were glia cells, we stained the adjacent slides with either IL-20 or anti-GFAP Ab (a marker of glia cells). The IL-20-positive cells were also positively stained with the anti-GFAP Ab in the adjacent slides, suggesting that IL-20 was expressed specifically on glia cells in the brain (Fig. 4*E*). These results demonstrated that hypoxia up-regulated IL-20 expression in vivo and that glia cells were a major source of IL-20 in the brain after ischemic injury.

Induction of cell proliferation and signal transductions by IL-20 in GBM8901, a human glioblastoma cell line

Reactivated glial cells proliferate and release multiple cytokines and chemokines in the ischemia-injured brain (36). Immunohistochemical staining showed that IL-20 was up-regulated in glia-like cells after ischemic brain injury (Fig. 4*C*). Thus, we further tested the possibility that glial cells could be the source and target of IL-20 and contribute to the inflammatory response by altering the expression levels of proinflammatory cytokines and chemokines. We used GBM8901 glioblastoma cells in the in vitro assays. RT-PCR showed that GBM8901 cells expressed endogenous IL-20 mRNA and IL-20 receptor subunits (IL-20R1, IL-22R1, and IL-20R2), which indicated that GBM8901 cells might be a target of IL-20 (Fig. 5*A*). Incubating hypoxic GBM8901 cells (1% O_2) significantly up-regulated their IL-20 mRNA (Fig. 5*A*) and protein (Fig. 5*B*) levels ($p < 0.05$). The promoter assay also showed that the luciferase activity of the IL-20 promoter construct pE was enhanced by $CoCl_2$ or hypoxia in GBM8901 cells and was abolished

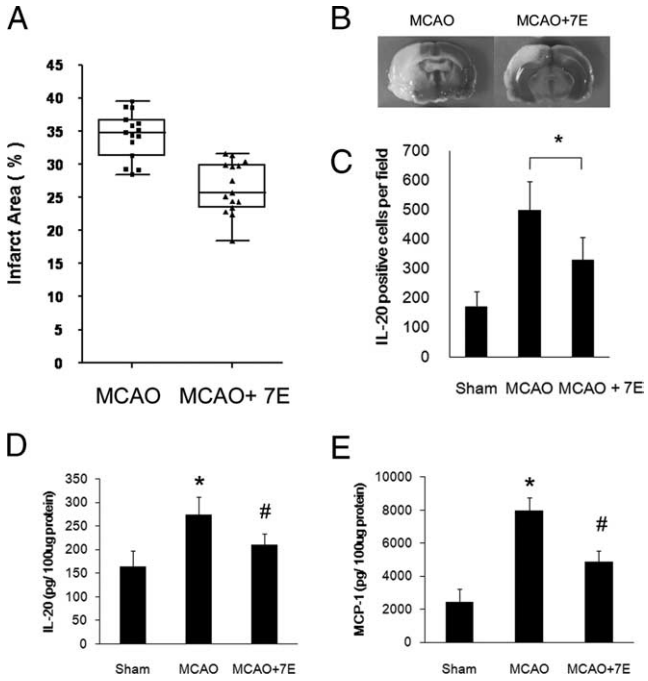

FIGURE 7. Amelioration of ischemic brain infarction in rats after MCAO by anti-IL-20 mAb 7E. *A*, Rats ($n = 15$ each) underwent 90 min of MCAO and ischemic reperfusion was i.v. injected with PBS or anti-IL-20 mAb (10 mg/kg). The brain slices were analyzed for the lesion areas 3 days after MCAO using TTC staining. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the 10th and the 90th percentiles. Lines inside the boxes represent the median. Differences between the 7E and control groups are statistically significant ($p < 0.05$). *B*, Representative lesion areas analysis of the rat brain slices using TTC staining. *C*, Quantification of the number of IL-20-positively stained cells in the ischemic-infarcted lesion in the right MCA cortex and adjacent penumbra.*, $p < 0.05$ compared with MCAO treatment. Brain tissues after MCAO and treatment with 7E were collected and analyzed using ELISA for IL-20 (*D*) and MCP-1 (*E*). *, $p < 0.05$, compared with sham control. #, $p < 0.05$, compared with MCAO treatment.

by mutation of the core sequence of HRE1 (Fig. 5*C*), which was consistent with the promoter results in HEK293 cells (Fig. 2*F*). Thus, IL-20 regulation by hypoxia is a common phenomenon in different types of cells.

Western blotting also showed that IL-20 induced phosphorylation of STAT3, ERK1/2, and AKT (Fig. 5*D*). In addition, IL-20 promoted the proliferation of GBM8901 cells, and the anti-IL-20 mAb 7E neutralized IL-20-induced cell proliferation in the MTT assay (Fig. 5*E*). We also analyzed the inhibitors of signal transduction molecules on IL-20-promoted cell proliferation. the ERK1/2 inhibitor PD98059 and the JAK2 inhibitor AG490 inhibited IL-20-induced cell proliferation. These results demonstrated that GBM8901 glioblastoma cells were a source and target of IL-20 and that IL-20 targeted GBM8901 cells and promoted cell proliferation through STAT3- and ERK-dependent pathways (Fig. 5*F*).

Up-regulation of proinflammatory cytokines and chemokines in glial cells by IL-20

Inflammation is associated with brain injury after ischemic stroke. Proinflammatory cytokines and chemokines such as IL-1β and IL-8 contribute to the inflammatory response in the infarcted lesion (11, 14). To test whether IL-20 alters the expression levels of cytokines and chemokines, we treated GBM8901 cells with IL-20

alone or IL-20 combined with IL-1β. IL-20 alone induced the production of IL-1β, IL-8, and MCP-1, but it inhibited the production of MIF (Fig. 6). Anti-IL-20 mAb 7E neutralized the effect of IL-20, which indicated that the activity is specific. IL-1β alone induced the production of IL-8, MCP-1, TNF-α, and IL-6. Combined treatment with IL-20 and IL-1β further increased the production of IL-8, MCP-1, TNF-α, and IL-6. These results suggested that IL-20 might contribute to the inflammatory response in combination with IL-1β to increase the production of proinflammatory cytokines and chemokines in glial cells after ischemic stroke.

Amelioration of brain infarction after MCAO by IL-20 Ab

We used anti-IL-20 mAb 7E to evaluate the role of IL-20 in the progression of ischemia-induced brain infarction. 7E (10 mg/kg) was i.v. injected immediately after 90 min of MCAO and ischemic reperfusion. Rats were sacrificed after 72 h of MCAO for brain sampling and lesion area analysis. Lesion areas were analyzed using TTC staining. The median infarct areas were 34.3 (25th to 75th percentiles, 36.8–31.5) for the PBS-treated MCAO control and 26.4 (25th to 75th percentiles, 30.1–23.8) for the 7E-treated MCAO groups (Fig. 7*A*). These findings indicated that the severity of infarct areas could be ameliorated after treatments with 7E ($p < 0.05$). Representative analysis of the lesion areas using TTC staining showed reduced infarct areas in 7E-treated MCAO groups (Fig. 7*B*). Furthermore, immunohistochemical staining showed that the number of IL-20 positively stained glia-like cells was reduced in 7E-treated MCAO brain than that in MCAO control (Fig. 7*C*). In addition, ELISA of the brain lysates showed that the up-regulated protein levels of IL-20 and MCP-1 after MCAO were reduced by treatment with 7E ($p < 0.05$; Fig. 7, *D* and *E*). These results suggested that 7E reduced ischemic brain infarction areas through inhibiting the expression of IL-20 and MCP-1 and the proliferation of glial cells after ischemic brain injury.

Discussion

We showed that $CoCl_2$-mimicked hypoxia up-regulated IL-20 expression in HaCaT and HEK293 cells, primary chondrocytes, and monocytes, which suggested that this mechanism operates in different types of cells. We also identified the TSS with three rounds of 5′ RACE and found that there were 1013 bp in the 5′ untranslated region. Our analysis of the human *il20* gene promoter region showed pC (−996/+595) and pD′ (−441/−8) fragments that supported luciferase activity at a level 4-fold greater than that of the negative control. The fluctuation of promoter activity in pA, pB, pC, pD, and pE may be due to some repressor or enhancer sequences located in these regions. The fragment from −8 to +595 containing HRE1 (+372/+376) showed no regulatory activity other than hypoxic-response activity. The 27 bases of the HRE-binding matrices, HRE1, also showed promoter activity increased by $CoCl_2$-mimicked hypoxia, which indicated that the minimal functional sequence of the HRE-binding matrix is sufficient for hypoxia-regulated IL-20 expression.

HIF-1α is the major mediator in cells in response to hypoxia. Our results showed that the up-regulation of IL-20 by hypoxia is correlated with the protein level of HIF-1α using the HIF-1α inhibitor 2ME2. A recent study (37) showed that 2ME2 treatment in a rat MCAO model reduced the brain-infarcted area, which suggested that the HIF-1α-mediated hypoxic response is a detrimental effect in ischemic brain injury. In the ischemia-injured brain, HIF-1α expression has been reported in both neurons and glial cells (38–40). However, HIF-1α expression in neuron and glial cells had different outcomes after an ischemic brain injury (41). The loss of HIF-1α function in neurons reduced neuronal viability after hypoxia, whereas the selective loss of HIF-1α function in astrocytes protected neurons from hypoxia-induced neuronal death, suggesting that HIF-1α has a cell type-specific and bifunctional role in astrocytes and neurons (41). Therefore, it is interesting to further clarify the significance, for neuronal functions, of the HIF-1α-mediated up-regulation of IL-20 in glial cells after ischemic stroke.

Excessive gliosis after an ischemic brain injury creates a formidable barrier to axonal growth and neuronal regeneration. In addition to a negative role in neurite outgrowth (42), the protective role of reactive astrocytes in brain ischemia is important for isolating necrotic areas and preventing the spread to healthy neighboring tissue of cytotoxic products released from dead cells (43, 44). Reactivated glial cells proliferate and release multiple cytokines and chemokines to recruit neighboring astrocytes or peripheral leukocytes into the inflamed sites (36). IL-20-promoted proliferation and cytokine production in GBM8901 glioblastoma cells might contribute to the reactivation of resting glial cells and trigger gliosis in ischemic brain tissues. Bacterial endotoxin induces IL-20 expression in glial cells (45). We found that hypoxia up-regulated IL-20 expression in vitro. In vivo, we detected IL-20 in glia cells 3 days after MCAO in the peri-infarcted lesion. Interestingly, GFAP expression (a specific marker for astroglia) also peaked 3 days after ischemia in a rat MCAO model (46). IL-20 up-regulation might be associated with reactive gliosis after an ischemic brain injury. The significance of IL-20 in gliosis in vivo awaits further studies. We also detected up-regulation of IL-20 in serum 3 days after MCAO. It has been shown that there was blood-brain barrier damage and leakage after ischemic stroke (47). In addition, stroke also induces a rapid activation of peripheral immune system (48). The source of the elevated IL-20 expression in serum might be hypoxia-activated glia cells, peripheral monocytes, or other unidentified cells activated after an ischemic brain injury. Therefore, it is worthwhile to evaluate whether the elevated serum level of IL-20 is associated with the brain infarct area and its clinical outcomes.

IL-20 is induced when IL-1β modulates p38 MAPK and the NF-κB-dependent pathway (49). NF-κB mediates ischemia-induced neuronal death via IκB kinase (50). Elevated IL-1β mRNA expression occurs within 15–30 min after ischemia (10) and elevated IL-1β protein expression occurs a few hours later (51). We found that IL-1β-induced IL-20 occurs in GBM8901 cells, which suggested that IL-1β acted as an important modulator for inducing IL-20 expression. In addition, IL-20 up-regulated the production of IL-1β production and combined treatment with IL-20 and IL-1β increased the production of other proinflammatory cytokines in GBM8901 cells. Based on these observations, we conclude that IL-20 and IL-1β may act synergistically to promote tissue inflammation.

IL-20 also induced the production of IL-6, a major proinflammatory cytokine. The serum level of IL-6 correlates with brain infarct volume and stroke severity (52). IL-20 may contribute to the elevated serum level of IL-6 after ischemic stroke, the clinical significance of which requires further exploration.

MCP-1 and IL-8 are critical factors that regulate postischemic inflammation and induce not only leukocyte recruitment but also blood-brain barrier disruption and leukocyte adhesion to brain endothelial cells. Mice without the chemokine receptor CCR2 are protected against ischemia-reperfusion injury (53). Interestingly, the microarray data of this study showed that IL-20 mRNA was up-regulated after 5 days of MCAO, but IL-20 up-regulation was not detected in CCR2-deficient mice after MCAO (53). This observation suggested that MCP-1/CCR2-mediated signals might contribute to the induction of IL-20. Our previous studies (24, 27,

54) showed that IL-20 up-regulated MCP-1 expression in several types of cells. It is possible that IL-20 and MCP-1 act in a positively regulating loop during tissue inflammation.

The in vivo data showed that inhibition of IL-20 by the specific mAb significantly ameliorated the brain ischemic infarction in rats after MCAO. The reduction of infarction areas by 7E treatment was associated with the reduced protein levels of IL-20 and MCP-1 and the number of IL-20-postive glia cells, which indicated that IL-20 was involved in the progression of ischemia-induced brain injury through regulation of glia cell activation and proliferation.

Cytokines such as IL-1β, TNF-α, IL-6, and IL-10 are involved in various inflammatory diseases. As a general response to tissue injury, inflammation is ubiquitous. Separating out the different causes of the inflammatory response is important because of the possible therapeutic implications. Our study identified IL-20 as a novel hypoxia-responsive factor that was up-regulated in glial cells after experimental ischemic stroke and that mediated cell proliferation, signal transduction, and cytokine production. Our findings provide evidence that IL-20 is involved in the pathogenesis of ischemic stroke and suggested that the antagonist of IL-20 might have therapeutic potential.

Acknowledgments

We thank Prof. Lin Mao-Tsun (Chimei Medical Center, Tainan, Taiwan) for help in establishing the MCAO model.

Disclosures

The authors have no financial conflict of interest.

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