Abstract. Immunological alterations have been reported to be involved in glioma, the most common malignant disease of the adult brain. Our recent study identified higher levels of IL-17 in glioma specimens. The present study investigated the role and possible mechanisms of IL-17 in glioma tumorigenesis. Human IL-17 cDNA was cloned and inserted into the eukaryotic pEGFP-N1 expression vector, which was used to transfect the glioma U87MG cell line, resulting in a high level of IL-17 expression in these cells. The cells were then transfected with IL-17 (pEGFP-N1-IL-17-U87MG) or mock (pEGFP-N1-U87MG) vector or left untransfected (U87MG) and subcutaneously inoculated into the right flank of nude mice. The results revealed that the pEGFP-N1-IL-17-U87MG cells grew more rapidly in the early stages (P<0.05, determined on day 32 post-inoculation compared with the other two groups). Quantitative (q)PCR detected higher mouse (m)CD31 mRNA levels in the IL-17-transfected group (P<0.01) compared with the mock-transfected and untransfected groups. IL-17 transfection altered the mRNA expression of a panel of molecules that are associated with immunity and inflammation in U87MG cells in vitro. An effect of the vector was identified, whereby the mock transfection strongly inhibited cell growth in vivo and dramatically altered the mRNA levels of multiple molecules in the cell culture in vitro compared with the untransfected cells. The present study confirmed that IL-17 overexpression may enhance glioma cell growth in vivo, which may be associated with accelerated angiogenesis. IL-17 overexpression may also alter the cellular mRNA expression of immune-related molecules.

Introduction

Glioma is the most common malignant disease of the adult brain. The outcome of patients with glioma is poor, mainly due to the diffusion of the tumor into the brain parenchyma (1). Certain immunological dysfunctions have been identified in glioma, including elevated immunosuppressive factors (2-5), reduced total lymphocytes (6), an imbalance in T helper (Th) subsets (7-14) and the infiltration of immunosuppressive microglia and macrophage cells (15). The immunosuppressive mechanism causes patients to be incapable of eradicating tumor cells and results in the anergy of certain immunotherapies. Therefore, the identification of the role of immune regulatory factors in glioma is significant for obtaining an understanding of the tumorigenesis mechanism and identifying a new therapeutic strategy for this malignant disease.

IL-17 is a main effector cytokine of Th17 cells and has become a topic of interest following the identification of Th17 in immunology (16,17). IL-17 has been examined in immunology, including autoimmunity, infection, transplantation, allergy and tumors. IL-17 has been shown to promote tumorigenesis via certain mechanisms, including the upregulation of angiogenesis-related molecules, vascular endothelial growth factor (VEGF) and CD31, the activation of the IL-6-STAT3 signaling pathway, the downregulation of IL-12Rβ2, thus impairing Th1 function, and the suppression of cytotoxic T lymphocytes (CTLs), causing them to lose their cytotoxic effect via co-operation with CD8 (18-20).

Our recent study identified that IL-17 was expressed at a higher level in glioma tissues compared with trauma tissues (21). Other studies have also demonstrated that IL-17 or Th17 are expressed at higher levels in glioma (22,23). To further explore the role and progress of IL-17 in glioma tumorigenesis, human IL-17 cDNA was cloned and packed...
into the eukaryotic pEGFP-N1 expression vector. The recombinant pEGFP-N1-IL-17 vector was then stably transfected and expressed in the glioma U87MG cell line. The present study investigated the role of IL-17 in promoting glioma tumorigenesis.

Materials and methods

Recombinant vector and gene amplification. The pEGFP-N1 plasmid was provided by the Institute of Military Medicine Science (Beijing, China) and re-confirmed by sequencing. Peripheral blood (2 ml) was drawn from the peripheral vein from a patient with idiopathic thrombocytopenic purpura (ITP) at the Huashan Hospital (Fudan University, Shanghai, China), according to the Sample Manipulation Guidelines of Huashan Hospital. This study was approved by the ethics committee of Huashan Hospital, Fudan University. Written informed consent was obtained from the patient. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll centrifugation at 400 x g. The PBMCs were cultured in RPMI-1640 medium supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 10% heat-inactivated fetal calf serum (Gibco, Carlsbad, CA, USA). The PBMCs were stimulated for 4 h with 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO, USA) and 1 µM mononycin in the presence of 10 µg/ml brefeldin A (Alexis Biochemicals, San Diego, CA, USA). mRNA was extracted and cDNA was synthesized using quantitative (q) PCR with primers containing enzymatic digestion sites for BamHI and SalI, according to the manufacturer's instructions. The primers corresponded to NCBI Reference Sequence (NM_002190.2) forward, 5'-CAG TCG ACG ACT CCT GGG AAG ACC TCA TTG-'3 and reverse, 5'-GG TGG ATC ATC CAT AAC CGG AAT ACC A -'3 and reverse, 5'-AGC ®uman glioma U87MG cell lines were identified by fluorescence and the positive clones were transferred into a 96-well plate with G418. The cells were diluted to 1 cell/well in a 6-well plate, and 20 µg pEGFP-N1-IL-17 or pEGFP-N1 plasmid were transfected into the eukaryotic pEGFP-N1-IL-17-U87MG and U87MG cells (5x10^6 cells of each type) were inoculated subcutaneously in the right flanks of the nude mice, with 10 mice in each group. The xenograft tumorigenesis effects were observed for the first time at 7 days post-inoculation and monitored once every 3 days. The tumor volumes were measured on days 32 and 35 post-inoculation. The mice were sacrificed on day 39 and the masses and volumes of the xenograft tumors and spleens were measured. Tumor volume (V) was calculated using the formula V = 4/3πr^3 (a and b are the long and short diameters of the tumor, respectively). The RNA of the tumor tissue was extracted and mouse (m)-CXCR2, -CD31, -matrix metalloproteinase 3 (MMP3) and -intercellular adhesion molecule-1 (ICAM-1) were qualified using qPCR. The conditions for the qPCR were 95°C for 30 sec, 95°C for 10 sec and 60°C for 30 sec for 40 repeats and 95°C-60°C-95°C for the melt curve observation. The primers that were used are listed in Table I. Each sample was tested in triplicate and the RNA of the target molecules were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results were calculated as 2^-△△CT.

qPCR determination of mRNA expression in vitro. In addition to using qPCR to detect the four mouse genes being expressed in the tumor tissues, the mRNA of a panel of molecules associated with immune and inflammatory responses in cells cultured in vitro were also detected using this technology. The molecules that were detected were: The chemokines, CXCL1, CXCL5, CXCL8, CXCL10, CXCL11, monocyte chemoattractant protein-1 (MCP-1; CCL2), regulated on activation, normal T cell expressed and secreted (RANTES; CCL5), CCL20, CCR4 and CCR6; the immunology regulation factors, β2-MG, PD-L1, prostaglandin E2 (PGE2), transforming growth factor (TGF)-β, IL-6 and STAT3; and the intercellular matrix molecules, MMP3, ICAM-1 and VEGF. The primers for the detection of these genes were human sequence-specific while those that were used for the detection of the genes in the tumor tissues were mouse-specific (with ‘m’ prefixing the gene name in this study). The primer sequences are listed in Table I. qPCR was performed using the conditions that were described previously.

Statistical analysis. The statistical analysis was performed using SPSS 11.5 (SPSS, Inc., Chicago, IL, USA). The data
were analyzed by ANOVA and logarithmic transformation was used if necessary. P<0.05 was considered to indicate a statistically significant difference.

Results

Human IL-17 is successfully expressed in U87MG glioma cells. IL-17 cDNA (NCBI reference sequence, NM_002190.2) was synthesized using RNA that was extracted from PBMCs of an ITP patient. Sequencing and restriction enzyme digestion were used to confirm the successful packages of IL-17 cDNA into the pMD19-T and pEGFP-N1 vectors. The results revealed that the IL-17 cDNA was inserted into the multiple cloning site (MCS) of the pEGFP-N1 vector. The target gene fragment (IL-17 cDNA) was 468bp and the sequence fully corresponded to that in Genbank (Fig. 1).

The cells that were transfected with pEGFP-N1-IL-17 and pEGFP-N1 were selected using 200 µg/ml G418. Following 10 days, the cells were diluted to 1 cell/10 µl. Subsequent to forming an expansive culture, the cells were identified using a fluorescence microscope and IL-17 mRNA and protein expression was detected by qPCR and an enzyme-linked immunosorbent assay (ELISA). The results revealed that the U87MG cells that were transfected with pEGFP-N1-IL-17 and pEGFP-N1 exhibited fluorescence, indicating that the vector was expressed successfully in those cells. Notably, the pEGFP-N1-IL-17-U87MG cells demonstrated a significantly higher level of IL-17 mRNA and protein compared with the pEGFP-N1-U87MG and U87MG cells (P<0.001; Fig. 2).

IL-17 overexpression promotes U87MG tumorigenesis in nude mice with elevated CD31 in tumor tissues. pEGFP-N1-IL-17-U87MG, pEGFP-N1-U87MG and U87MG cells (5x10^5) were subcutaneously inoculated into the right flanks of the nude mice. At 7 days post-inoculation, neoplasms became visible and the tumor sizes were monitored every 3 days. At 32 days post-inoculation, the sizes of the neoplasms in the pEGFP-N1-IL-17 group were larger than those of the pEGFP-N1-U87MG (P<0.05) and U87MG (P<0.05) groups. At 35 and 39 days, the tumor volume of the former group remained larger than the latter two groups, but had no statistical significance with the U87MG group, indicating that IL-17 may have accelerated tumor growth at an early stage (Fig. 3).

To explore the possible mechanism underlying the differences in tumor growth among the three groups, the mRNA

| Table I. Primer sequences. |
|---------------------------|
| Gene          | Forward primer | Reverse primer | Product size, bp |
|---------------|----------------|----------------|-----------------|
| GAPDH         | TTCGACAGTCAGCCGCCTATCT | GTGACCAGGGCCCAATAACG | 115 |
| MCP-1         | GGCTGAGAATCAAACCGAAACATC | TGACTGGGGCATTTGACAT | 158 |
| RANTES        | GCTGCTTTTGGCTACATTGCC | ACTTGCGGTGTTTGGTGTTG | 118 |
| CXCL1         | GAAGCTGAATGGCTCCCCGAC | GCCACAGTTGACCTTTGCC | 175 |
| CXCL5         | GCACGCGCTCTTCTGACACT | ACGCAGACGCTCTCTACAC | 169 |
| CXCL8         | AACCTTCAGAGAGCAAGCAGCAGCACC | GCCACTCGCTGGCAAAACCTGCA | 173 |
| CXCL10        | TGAGCTGACAGAGGTTGACGTTACC | TGCTGATGAGTACGCTTACGTA | 139 |
| CXCL11        | GCCTTGCGGTGATATTGTGTCG | CTGCTTTTACCCCGAGCT | 94 |
| CCL20         | CAGTGGCTGCTACTCCACTCTC | TGCCGCTGTAAGGCCCCAACATTA | 112 |
| CCR4          | GCTGGACTGGTGGCACCTCAGCA | AAGGGCTCTCTCTGAGGCTT | 101 |
| CCR6          | TATTGAGTCACCTCTTCTTTCTT | ACTGGAGTGGCAAAACATCGTGA | 147 |
| PD-L1         | TGTTTGTTGCGGACCCACTACAG | GGTTAGCCTCTAGCCGTACA | 129 |
| STAT3         | AGGAGCATCCTGAAGCTGGACCCA | GAGGGTCTACAGACCTTACCAT | 163 |
| β2-MG         | AGTATGCCTGGCGTGATTGCC | GGGCATCTTTAAACCTCCTCA | 100 |
| PGE2          | GTTGGTTGAGGAGGGCCGCG | GGGAGCAGCTTTGGGTCAGG | 173 |
| IL-6          | AAGCCAGAGCTGTGCCAGATGA | TGGCTCTTGTCCTGACGCTT | 136 |
| TGF-β         | GGCACTGCTTTGGCAACCAA | GGGCGCCGGTGATATGGTGT | 220 |
| ICAM-1        | AGTGCAGCTGACTGCTCTTCT | TGCTGAGTACGCTTGAGCTC | 137 |
| VEGF          | GGTGCTGCGTGCTCTGCTTAA | GGCTCTGATGCTGACCTTCACT | 194 |
| MMP3          | GTAAGATTAAGCCTAGGGAATGAAA | ACAGGACCAGCTCTTTTCTCC | 199 |
| mCXR2         | GTTCAACAGCCTCTGGACAGCT | TGCGACATGAGGACCTTACGT | 207 |
| mCD31         | GAGAAGCCCAACAGCCATTACGG | GAGCTCTCGTTCTCTTGTGGA | 151 |
| mMMP3         | GTGTGCTCATCTCCACTCCATTTG | TAGTTGAGGAGTGCTAAGCTTC | 211 |
| mICAM-1       | TGCCCTTCGCTGTGCCTTCAAC | AGTCTCCAGCCAGGCTGA | 196 |

*Gene names that are preceded by ‘m’ indicate that the primer sequence is mouse-specific. Otherwise, the primers are human-specific. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation, normal T cell expressed and secreted (CCL5); PGE2, prostaglandin E2; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; ICAM-1, intercellular adhesion molecule-1.*
levels of mCXCR2, mICAM-1, mMMP3 and mCD31 were detected in the tumor tissues. The results revealed a higher mCD31 mRNA level in the pEGFP-N1-IL-17-U87MG group (P<0.01) compared with the other two groups, while mICAM-1 mRNA was higher in the pEGFP-N1-U87MG group (P<0.05) compared with the other two groups. The levels of mCXCR2 and mMMP3 mRNA were not significantly different among the three groups (Fig. 4).
IL-17 transfection alters the mRNA levels of a panel of immune/inflammation-related molecules in U87MG. To further understand the role of IL-17 in the behavior of U87MG cells, qPCR was used to detect the mRNAs for a panel of proteins that are associated with immune and inflammation responses, including intercellular adhesion, the intercellular matrix and chemokines. The expression of a series of molecules was altered in the pEGFP-N1-IL-17-U87MG, pEGFP-N1-U87MG and U87MG cells (Fig. 5).

Discussion

IL-17, as the main regulatory element of the emerging Th17 subset, has gained considerable interest. Our recent study identified a higher level of IL-17 in glioma tissue (21). The present study identified that the overexpression of IL-17 may accelerate the early-stage growth of U87MG glioma cells in vivo. The expression of IL-17 also altered the mRNA profile of immune/inflammation-related proteins when transfected into a cell culture in vitro.

In the present study, human IL-17 cDNA was inserted into the pEGFP-N1 plasmid and transfected into the glioma U87MG cell line. The success of the procedure was confirmed using gene sequencing, GFP detection and IL-17 mRNA and protein determination. The U87MG, pEGFP-N1-U87MG and pEGFP-N1-IL-17-U87MG cells were inoculated into nude mice. The pEGFP-N1-IL-17-U87MG group demonstrated accelerated tumorigenesis compared with the other two groups when measured at 32 days post-inoculation (P<0.05). On days 35 and 39 post-inoculation, the implanted tumors of the pEGFP-N1-IL-17-U87MG group were larger than those of the other two groups. However, the difference was not statistically significant. This result indicated that IL-17 was able to accelerate glioma growth, particularly in the early stage of tumorigenesis.

To identify the possible mechanism behind the accelerated tumor growth caused by IL-17 overexpression, mCXCR2, MMP3, mICAM-1 and mCD31 mRNA expression in the xenografted tumor tissues were analyzed using qPCR. mCD31 expression in the tumor tissues of the pEGFP-N1-IL-17-U87MG group was higher than in the other groups (P<0.01). This was consistent with the results of a study by Numasaki et al (24). The effect of early-stage tumorigenesis acceleration caused by the overexpression of IL-17 may be associated with the promotion of angiogenesis, which is consistent with the notion that the formation of new blood vessels is vital for the initial growth stage for solid tumors. However, in contrast with the results from the study by Numasaki et al, the present data did not include an elevation in mCXCR2 in the tumor tissues from the IL-17 overexpression group. This difference may have been due to the different tumor types that were used in the two studies. Alternatively, the angiogenesis-promoting effects of IL-17 may also be active through pathways other than CXCR2, as described by a number of studies (25-27). The present study highlights the fact that IL-17 may be a target for interference in tumor angiogenesis.

In addition to detecting the mRNA levels of several molecules in the tumor tissues, the mRNA level for a panel of molecules that are associated with the immune response and inflammation were also analyzed in the transfected cells in order to understand the alterations caused to the behavior of the U87MG cells by IL-17 in vivo. IL-17 was able to increase the levels of CXCL10, CCL20 and β2-MG. However, whether these changes are associated with the acceleration of the early-stage
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