Epidermal growth factor (EGF) promotes human malignant glioma invasion by mediating secretion of human cytomegalovirus infected monocyte-derived macrophages

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Human malignant glioma is the most aggressive brain tumor which lacks efficient therapies. Accumulating evidence indicates that human malignant gliomas are universally infected with human cytomegalovirus (HCMV). Recent studies demonstrate that tumor-associated macrophages (TAMs) density is associated with glioma grade. We hypothesize that virus affected the secretion of macrophages which infiltrated into glioma to promote the glioma cell invasion. Supporting this hypothesis, we showed that the secretion factor EGF had increased when HCMV infect macrophages. And the expression of the epidermal growth factor receptor (EGFR) on the surface of malignant U87 cells also up-regulated, indicating the infection of HCMV can impact the secretion factor EGF of macrophages and then activates EGFR of malignant glioma cells. We measured focal adhesion kinase FAK Tyr397 which is necessary for cell mobility. HCMV infected-macrophages can up-regulate the expression of FAK Tyr397 of malignant U87 cells. Together, these results provide evidences that EGF from infected-macrophage can activate EGFR of human malignant glioma and promote glioma cell invasion. In conclusion, these findings indicate that infected-macrophage with HCMV play an important role in the invasion of glioma and may act as a potential target to prevent its invasion.

Keywords: glioma; human cytomegalovirus; invasion; macrophage; microenvironment


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Introduction

Human glioma is the most aggressive brain tumor which lacks efficient therapies. Patient survival time is universally 12-15 months. Even having received radiation and chemotherapy, the majority of patients die within 2 years [1]. Accumulating evidence indicates that human malignant gliomas are universally infected with human cytomegalovirus (HCMV). HCMV is part of the β-subgroup of the herpesvirus family [2]. The seroprevalence of HCMV in the normal population is more than 80% whose persistent infection maintains its whole life. Over 70% adults have been infected all over the world. And HCMV is the cause of congenital nervous system disease in neonates. HCMV infection causes several human malignancies and morbidity in immunocompromised hosts, including tumor invasion,
inflammatory, immune regulation, etc. [3]. Recently, more evidences show that HCMV plays a potential role in oncogenesis [4]. Numerous reports show that HCMV proteins and DNA appear in human glioblastoma tissue samples [5, 6]. In malignant glioma specimens, immediate-early 1 (IE1) -72 protein (UL123), p52/76 kDa delayed-early protein, and pp65 tegument protein have been found [5-7]. And, the gene products of HCMV can change signaling pathways thus to alter cellular proliferation, migration, apoptosis, and transformation [8]. Previous reports have been obviously reported that epidermal growth factor receptor (EGFR) signaling in malignant glioma plays a significant role [9, 10] in malignant glioma. EGFR is a extremely important cellular target for HCMV-infected host cells its activation activates the signaling channel of downstream cells [9]. Given various reports shown that stimulated activation of EGFR in glioma can bring biology alterations [11, 12].

Macrophage is the most infiltrating cell type found in organs infected by HCMV [13, 14]. And the majority of malignant tumors recruit macrophage [15]. Recent study shows that tumor-associated macrophages (TAMs) take part in tumor microenvironment in promoting tumor initiation, cell migration and invasion [16, 17]. The clinical evidences show that brain macrophage accumulates in and around the glioma [16]. And abundant macrophage infiltration is a universal phenomenon in glioblastoma [18]. In the beginning of HCMV infection, monocytes play an important role in the latent viral infection [19]. However, the monocytes have a short lifespan and inability to support viral gene expression and replication. So infected-monocytes promote differentiation into long-lived macrophages that supports viral gene expression and replication of viral genes [19]. So we sought to investigate that macrophage with HCMV infection are associated with malignant glioma. Here we show that HCMV infection leads to change of macrophage secretion, and promote the invasion of glioma. Together, our data together provide insights into the secretion change of HCMV-infected macrophage which can modulate cell growth, survival, and invasion.

Due to the high prevalence of HCMV-infected macrophage and human malignant glioma in vivo, this study aims to investigate the important role of HCMV infected-macrophage in malignant glioma cell invasion.

### Materials and Methods

**Cell lines and cell culture.** Human glioblastoma U87 cell lines, Human embryonic lung fibroblasts (HELF), Human THP-1 monocyic leukemia cells were purchased from the Shanghai Cell Resource Center of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in a humidified atmosphere containing 5% (v/v) CO2 at 37 °C. U87 cells were maintained in HyClone™ MEM with 10% fetal bovine serum (FBS). Cultures of HELF were maintained in HyClone™ DMEM Ham’s F-12 (DF-12), supplemented with 10% FBS. THP-1 cells were grown in HyClone™ RPMI 1640 medium supplemented with 12% FBS. Having been induced by 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) in the medium for 48h, THP-1 differentiates into macrophage.

**Virus and virus infection.** HCMV AD169 (France Pasteur Laboratory, Paris, France) was propagated in human embryonic lung fibroblasts cells. In order to synchronize cells in G0, cells were starved in 0% serum at least 2h before virus infection. Then the inoculum was replaced with fresh inoculum. The cell supernatant was harvested at the indicated times and stored at -80 °C. Virus was titrated by plaque assays as described previously. All cell types were infected at a multiplicity of infection (MOI) of 5.

**Extraction of total RNA and reverse transcription-PCR (RT-PCR).** RNA was extracted by TRIzol reagent [Takara Biotechnology, China]. Manipulations were strictly executed by the manufacture’s protocol. RNA (1 µg) was subjected to reverse transcription with the RevertAid First Strand cDNA Synthesis kit [Takara Biotechnology, China]. Then PCR was performed according to the manufacturer's protocol. The cycling condition details as follows: Pre-denaturation at 95 °C for 5 min; denaturation at 94°C for 30 sec; annealing at 55°C for 30 sec; extension at 72°C for 1 min; and further extension at 72°C for 10 min. This PCR cycle was performed for 32 times. The primers were used in table I.

**Cell invasion assays in vitro.** Cell invasion assay was carried out using a 24 well cell culture insert (polyethylene terephthalate PET) with 8µm pore (3464, Corning, USA). Transwell assays were performed according to the manufacturer’s instruction. Briefly, THP-1 cells were treated with PMA (Sigma) for 48h to become monocyte-derived

### Table 1. Primers used in the present study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward 5'‑3'</th>
<th>Reverse 5'‑3'</th>
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<tr>
<td>IE</td>
<td>GCGCAATACATGAAAGATAAGGA</td>
<td>GATTGGTGTTGGCGGAACATG</td>
</tr>
<tr>
<td>UL99</td>
<td>GTGTCCTCATCCGGACTCG</td>
<td>TTCACAACGTCACCACCCAC</td>
</tr>
<tr>
<td>β-actin</td>
<td>TGGAACGGTGAAGGTGACAG</td>
<td>GGCTTTAGATGGCAAGGG</td>
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macrophages in the lower chamber. In the upper chamber 10% Mateigel was loaded into the chamber. Inoculate U87 cells on the inserted upper chamber. Cells invade to the bottom of the membrane for 8h and then fixed and stained. Images were captured by 10 × objective lens of inverted microscope. Invaded cells were counted with hemocytometer grid expressing as the average number of invaded cells per microscopic field.

**Western blot analysis.** U87 cells were grew to 80-90% confluency. To prepare cells, cold phosphate-buffered saline (PBS) washing it three times. Cells were lysed by 400 µl lysis buffer and 4 µl phenylmethylsulfonyl fluoride (PMSF, Beyotime Institute of Biotechnology, Shanghai, China). Cell lysates were centrifuged (Eppendorf Corporation, Germany) at 12 000× g for 5 min at 4 °C. Samples were resolved by 10% SDS-PAGE. The products were electrotransferred to PVDF membrane (Millipore, Billerica, MA, USA). PVDF membranes were incubated with anti-EGFR (Immunoway, USA), anti-FAK and anti-FAK Tyr397(Santa Cruz, USA) overnight at 4 ℃. After being washed three times with TBST, membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (Bioss, Inc.) for 2h at room temperature. Chemiluminescent signals were produced by the SuperSignal West Pico Trial Kit (Thermo Fisher Scientific Inc.).

Blots were imaged using the Vilber Lourmat imaging system (Vilber Lourmat Corporation, Torcy, France).

**ELISA assay.** The secretion change of HCMV-infected macrophages was determined using the enzyme-linked immunosorbent assay (ELISA) assay. Macrophages were infected or mock infected HCMV. Sample supernatants were collected at indicated times. Then samples were analyzed by ELISA followed the manufacturer’s instruction (Boster, China). Experiments were repeated independently three times.

**Statistical analysis.** The data were analyzed using SPSS software. Statistical analysis was performed by Student’s t-test. P<0.05 was considered statistically significant.

**Results**

1. HCMV establishes replication in infected macrophages

THP-1 human monocytic cell line can be induced into macrophages by being treated with 100ng/ml PMA for 48h *in vitro*. Lamellipodium induced in monocytes was the characteristic of macrophage (Fig. 1A and 1B). As HCMV permissive cells, macrophages play an important role in viral transmission. Macrophages become an ideal cell model for studying HCMV replication in the host.

Cells were infected HCMV were continuing 48h post-infection (p.i.) for viral gene expression. Compared with monocytes, which can not fulfill viral replication, infected macrophages completely express viral gene from viral immediate-early ("IE") to late ("UL99") HCMV transcripts. Total RNA extraction from monocytes and macrophages or HELF was analyzed by RT-PCR. The cellular β-actin was used as a control for PCR.

2. HCMV promotes the expression EGF of macrophages

HCMV infection induces various changes of secretion. To further examine the relationship between HCMV infection and the secretion change of infected macrophage, we examined EGF that its overexpression always associated with GBM procession. The macrophage cells were infected and mock infected with HCMV. Supernatant patterns were harvested at 0, 24, 48, 72h p.i. and analyzed by ELISA (Fig. 2). Obviously, the secretion of EGF increased following HCMV infection. As macrophage cells infected with HCMV, the EGF level was greatly enhanced (P<0.05; between 0 and 72h). This observation indicated that HCMV infection
creates a subtle microenvironment to promote the EGF expression of macrophages.

3. HCMV-infected macrophages upregulate EGFR activation of U87 cells

Previous studies have demonstrated that EGFR plays a key role in human malignant glioma. EGFR activation is closely associated with tumor cell growth, migration, and invasion. HCMV infection macrophages changes secretion factor EGF that activates U87 cell surface EGFR. Therefore, we co-culture macrophages and U87 cells in 6-well plates with inserts. Firstly, primed monocytes cells were cultured in the upper chamber and treated with PMA 48h to induce macrophage. Then U87 cells were seeded in the lower chamber. Macrophage cells were infected or mock infected HCMV for 0h, 24h, 48h and 72h. Sample proteins were harvested from U87 cells and analyzed by western blot (Fig. 3). It showed that EGFR protein levels that from U87 co-culture with HCMV infected macrophages were increased when compared with the control. There were no obvious enhancements in other groups. These results indicate that infected macrophages activation results in up-regulation of EGFR expression in U87 cells. In addition, directly treated with EGF also up-regulate the level of EGFR, but it is lower than the group of infected macrophages. So we assume that HCMV infected macrophages change a lot of secretions, EGF just one of them.

4. HCMV infection of macrophages promotes U87 invasion

An important hallmark of aggressive human malignant glioma is their high capacity to invade. To determine whether direct interactions between infected macrophages and glioma cells can regulate glioma invasion, we performed a Mateigel invasion assay (Fig. 4A~F). As shown in it, treatment of HCMV-infected macrophages with U87 cells dramatically increased U87 cells invasion. In addition, we analyzed that FAK Tyr397 that is required for integrin-mediated cell motility. The result also proved the same result that FAK Tyr397 expression level of HCMV infection was higher than mock infection (Fig. 4G). Those results indicated that HCMV infected macrophages promote U87 cells invasion.

**Discussion**

The relationship between virus infection and tumor progression has been generally proved. Virus infection can promote neoplastic transformation. Increasing evidence shows a strong association between HCMV and malignant gliomas [1, 2]. HCMV mediates host cells transcription and secretion to avoid its diffusion in order to establish a lifelong latent infection. Glioblastoma (GBM) is the most prevalent malignant tumors and lack of therapeutic methods [2]. Increasing evidences show that HCMV proteins and oligonucleotides had been detected in the GBM samples [5, 24]. More than 90% of human malignant gliomas exist in HCMV infection [1]. Therefore, it is very important to understand how HCMV effects and mediates human being cells.

HCMV has the ability to modify the host environment to benefit their replication. Therefore, HCMV induces differentiation from monocyte to macrophage as a strategy for viral spread and persistence [13, 19, 20]. HCMV infects host hematopoietic system, monocytes are deemed as the primary cell type [21]. HCMV viral DNA is found in peripheral blood.
monocytes rather than in lymphocyte or B and T. HCMV infects monocytes in the blood and organs, but monocytes do not express HCMV gene expression and replication \[7, 19, 22\]. This phenomenon enforces HCMV alters the non-productive environment in order to complete viral multiplication cycle and spread widely. Therefore, HCMV induces monocyte-derived macrophage that acquires longer lifespan and better capacity to express viral gene \[23, 25\].

Recently, growing evidences implicate that abundant TAMs enrich in GBM \[18\]. TAMs play role to support tumor and may promote GBM tumor progression. Our study to investigate the relationship between the HCMV infection and human malignant glioma cells, especially targeted HCMV-infected macrophages. And to understand how virus changes the host cell microenvironment.

Our results demonstrated that HCMV infection of macrophages promotes U87 invasion in vivo. To determine other biological changes in the microenvironment of infected macrophages and glioma cells, we also examined the expression of EGFR that usually plays an important role in GBM progression. The results show that HCMV infection macrophages upregulate EGFR activation of U87 cells (Fig 3A). We also found an interesting phenomenon; the expression of EGFR does not continuously increase. When it arrive a peak, the expression of EGFR will subtly reduce (Fig 3A and B). We assume that this phenomenon associate with the HCMV infection mechanism.

In conclusion, we found one of the reasons in regard to the mechanism of the high capacity invasion of human malignant glioma. Those results shed light on the influence of HCMV infection on glioma cell invasion. HCMV may act as a potential therapeutic target.

**Conflicting interests**

Rui Zhou, Dong-meng Qian, Xiao-min Hua, Ming Hu, Hao Chen, and Bin Wang declare that they have no conflict of interest.

**References**