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PROMOTION OF *IN VITRO* GROWTH OF HUMAN MEDULLOBLASTOMA CELLS BY EXOGENEOUS IL-6

Liu Jia[1] 刘佳 Li Hong[1] 李宏 Hamou Marie-France[2] Nicolas de Tribolet[2]

1 Division of Molecular Biology, Shenyang Medical College, Shenyang 110031
2 Laboratory of Tumor Biology and Genetics, Service of Neurosurgery, University Hospital (CHUV), 1011 Lausanne, Switzerland

***In vivo* and *in vitro* expression of IL-6 and its signal transducer genes, IL-6R and gp130, in human medulloblastoma cells were investigated by the approaches of molecular biology and cellular immunology. The results revealed that 12 out of 13 samples examined were found to express IL-6R and gp 130, but none of them showed IL-6 expression. Then, the potential effects of exogenous IL-6 on the proliferation of medulloblastoma cell line, Med-3 were evaluated, which showed that IL-6 could enhance cell outgrowth dramatically. Our data thus for the first time demonstrate the important role of IL-6 as paracrine growth factor in the proliferation of medulloblastoma cells.**

Key words: Interleukin-6, Proliferation, Medulloblastoma cells

Medulloblastomas are malignant brain tumors which occur predominantly in childhood and has a poor prognosis because of its high proliferating potential and strong tendency of dissemination.[1-3] Although intensive investigations have been done in different aspects during the past decades, little is known regarding the genetic, especiall immunogenetic event(s) leading to its malignant transformation.[4,5]

Interleukin-6 (IL-6) has been known to be a multifunctional cytokine exerting its effects on the induction or inhibition of differentiation and proliferation of hematopoietic and some solid tumor cells, which largely depend upon the type of target cells.[6-8] In human brain tumors, a constitutive *in vivo* and *in vitro* expression of IL-6 genes was found in most fresh glioblastomas and established cell lines, and this is thought to be important in the establishment and progression of neoplastic transformation and/or in the complex interactions of glioma cells and immune system *in vivo*.[9] However, so far, no data concerning the status and functiuonal role(s) of this cytokine in medulloblastomas has yet been reported. In order to address this issue, we checked IL-6, and its signal transduction gene expression in medulloblastomas by different approaches and investigated the biological effects of exogenous IL-6 on *in vitro* cell growth.

MATERIALS AND METHODS

Samples

Twelve medulloblastoma specimens (Med-260, -313, -461, -543, -599, -622, -645, -753, -720, -737,

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-745 and -848) were collected from the operation room, snap/frozen in liquid nitrogen within two hours after removal and stored at -70°C until use. The established huamn medulloblastoma cell line, Med-3[10] was cultured in Eagle's minimal essential medium (MEM) supplemented with 10% FCS. The general background of the patients from whom the tumors are taken is listed in Table 1.

Table 1. Summary of RNA and protein analyses

Patients' No.	Lineage Marker		Gene Expression		
(Sex, Age)	GFFAP	Synaptop	IL-6*	IL-6R	gp 130
260 (M, 21)	+	+	–	+	+
313 (M, 33)	+	–	–	+	+
461 (M, 15)	+	+	–	+	+
534 (M, 21)	+	–	–	+	+
599 (M, 30)	–	+	–	+	+
622 (M, 8)	+	–	–	+	+
645 (F, 36)	+	–	–	–	–
720 (M, 34)	+	+	–	+	+
737 (F, 37)	+	+	–	+	+
745 (M, 9)	+	+	–	+	+
753 (M, 25)	+	–	–	+	+
848 (M, 32)	+	+	–	+	+
Med-3 (M, 24)	–	+	–	+	+

RNA Isolation And Hybridization

Total cellular RNA was extracted from the tumor samples by the method of Chomczynski, et al. 15 µg of RNAs from each sample were separated by electrophoresis on an 1% formaldehyde agarose gel, blotted onto Hybond-N^+ nylon filter (Amersham, UK) and hybridized with an α-^{32}P dATP labelled cDNA probe of human IL-6[9] overnight. The filter was washed twice in 2 × SSC/0.1% SDS for 15 minutes at room temperature, in 1 × SSC/0.1% SDS at 65°C for 30 minutes and in 0.2 × SSC/0.1% SDS for 15 minutes at room temperature, then exposed to Kodak XAR film at -70°C for 3 days. An established glioblastoma cell line, LN-428, was used as positive control for IL-6[5] and β-actin was utilized as a quantitative control for the relative amount of RNAs loaded onto the gel.

RT/PCR-Southern Blot Analysis

To confirm the results from Northern analysis, RT/PCR was performed on total RNA samples from medulloblastomas with the primers of IL-6, IL-6R and gp 130. The primer sequences are given as follows:

IL-6 5' ATG AAC TCC TTC TCC ACA AGC GC
3' G AAG AGC CCT CAG GCT GGA ATG
IL-6R 5' C ATT GCC ATT GTT CTG AGG TTC
3' A GTA GTC TGT ATT GCT GAT GTC
gp130 5' GAA GAA AAT GAG GTG TGA GTG AGT GGG ATG GTG
3' AGT TCT GAT GCC TTG AGT ATG GGA TGG AT

Briefly, 10 µg RNA of each sample was denatured at 65 ℃ for 10 minutes, then subjected to reverse transcription in 40 µl solution containing 8 µl of 5 × first strand buffer, 3 µl 100 mM DTT, 4 µl 5 mM $MgCl_2$, 8µl 0.25 mM dNTP mix, 2µl random primer and M-MLV reverse transcriptase (Gibco, USA). RT reaction was done at 37 ℃ for 60 minutes, followed by an incubation at 95 ℃ for 5 minutes to inactivate reverse transcriptase. With the use of the

same RT products, PCR amplification for IL-6, IL-6R, gp 130 and β-actin were performed in 50 μl solution containing 8 μl of RT solution, 5 μl 10 × PCR buffer, 2.4 μl 25 mM $MgCl_2$ 4 μl 1 mM dNTP mix, 1 μl upstream and 1 μl downstream primers and 0.5 μl AmpliTaq (Perkin Elmer, US). The conditions of PCR are as follows: 84 ℃, one minute, then 92 ℃, 40 seconds/60 ℃, 40 seconds/74 ℃, 90 seconds for 30 cycles or 36 cycles and 75 ℃, 5 minutes. For Southern blot analysis, the PCR products were resolved by electrophoresis on 1.2% agarose gels containing ethidium bromide, transfered to Nylon membrane and hybridized with radio-labelled probes specific for IL-6, IL-6R and gp 130 respectively by the method described previously.[11] Glioblastoma cell line, LN-428, was used as positive control for IL-6.

Immunocytochemical Staining

Immunocytochemical staining was carried out on frozen sections of tumor tissues or on coverslips of cultured cells using a monoclonal antibody (mAb) against human IL-6 (Genzyme, Combridge, MA). The staining procedures were performed as described previosly.[12] A glioblastoma tumor, G-727 was used as positive controls for IL-6. A small peice of normal cerebellar tissue excised from a young adult brain during autopsy was used as normal control. Supernatant of P3× 63Ag8, a γ-1-producing myeloma cell line was used as negative control. To evaluate any potential correlation between lineal differentiation of medulloblastomas and IL-6 expression, the samples were stained with mAbs against human glial fibrillary acidic protein (GFAP) (DAKO A/S, Copenhagen, Denmark) and neuronal marker, synaptophysin (DAKO A/S, Copenhagen, Denmark), respectively.

Cell culture And Treatments

The medulloblastoma cell line, Med-3 was cultured in Eagle's MEM supplemented with 10% FCS. 2 ml of the cells per dish were seeded at a concentration of 5 × 10^5/ml. after 2-day incubation, supernatants were collected and stored at 4 ℃ with the addition of 0.005% Tween 20 until use. Intracellular IL-6 were determined by staining the cells with mAbs against IL-6. IL-6 bioactivity in the supernatant was measured by the IL-6 bioassay using IL-6 dependent murine B-cell hybridoma 7TD1 cells.[13]

To evaluate cytokinetic changes of the cells under different treatments, the cells were seeded into 24-well plates in triplicate at 2 × 10^5 cells/well in 2 ml medium and allowed to attach overnight. The following day, different concentrations (0, 20, 30, 40, 50, 60U/ml) of rIL-6 was added into the medium, respectively. The cells were trypsinized, excluded by trypan blue and counted using a hemocytometer with 2-day intervals. the observation lasted for 12 days. Untreated cells and the cells treated with normal IgG were used as controls. The data were expressed as the mean value (counts/plate)± standard error of the mean. Each experiment was repeated at least for 3 times. For determination of influence of exogenous IL-6 in DNA synthesis of Med-3 cells, the cells were seeded into 96-well plates in triplicate at 2 × 10^4 cells/well in 0.2 ml MEME medium, 24 hours later, 40U/ml rhIL-6 was added and the cells were inculated for another 72 hours. 1 μci ^{3}H-Tdr (specific activity, 6.7ci/mmol) was added into each of the wells 16 hours before harvesting the cells and counting β radiation activity in the filters.

RESULTS

Northern blot hybridizations in Figure 1 revealed that when the filter was hybridized with a cDNA probe for IL-6, no positive signal could be detected in all of 13 medullablastoma samples while the glioblastoma cell line, LN-428, the positive countrol for IL-6 gave a clear hybridization band. RT/PCR-Southern blot analysis was then performed to exclude the possibility that IL-6 gene transcription could be below the treashold of detection by Northern blot analysis. As shown in Figure 2, IL-6R as well as gp130 was found to be expressed in 12 of 13 cases; in agreement with the results of Normthern blot analysis, IL-6 remained negative among the samples.

The results of immunocytochemical staining of medulloblastomas with anti-IL-6 mab showed thatnone of the medulloblastoma samples studied showed positive staining for IL-6 althogh a few of cells in the surrounding tissue were labelled sporadically (figure not shown). The labeling for differentiation markers of primitive neuroectodermal stem cells demonstrated that in 13 medulloblastoma samples studied, 11 contained GFAP positive cells and 8 contained synaptophysin positive cells. although mostof medulloblastoma tumors usually contained more than one cell subpopulations with the expression of GFAP, or synaptophysin or neither of them, no IL-6

protein could be detected in those three types of cells. The results of RNA and protein analyses are summarized in Table 1.

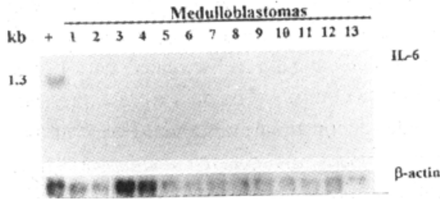

Fig 1. Northern blot analysis showing the sttus of IL-6 gene expression in human medulloblastoma tumors and cell line. No. 1-12 are the tumors with the case numbers of 848, 753, 745, 737, 720, 645, 622, 599, 543, 461, 313, and 260. No. 13 is an established cell line, Med-3. Glioblastoma cell line, LN-428 was used as positive control for IL-6. β-actin mRNA was used as quantitative and qualitative control.

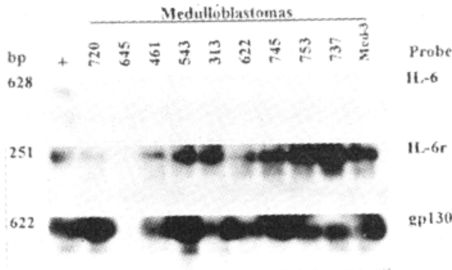

Fig 2. RT/PCR-Southern analysis of IL-6 and its receptor gene expression in medulloblatoma tumors/cell line. Glioblastoma cell line, LN-428 was used as positive control for IL-6. The cloned cDNAs of Il-6R and gp 130 were used as template for positive controls.

The presence of bioactive IL-6 in the supernatant of Med-3 cells was tested in the IL-6 dependent murine B-cell hybridoma 7TD1 proliferation assay. No bioactive IL-6 could be detected in 72 hour culture supernatants and the cells showed typical phenomina of apoptosis.[13]

The effect of exogenous IL-6 on Med-3 cell growth was investigated by incubation of Med-3 cells with different concentrations of concentrations of recombinant human IL-6 (0, 20, 30, 40, 50, 60U/ml) for 72 hours. It was shown that in comparison with Med-3 cells cultured normally, IL-6 could stimulate either the cell proliferation (Figure 3) DNAsynthesis (Figure 4) in a time related fashion. A concentration of rhIL-6 at 40U/ml gave the best promotory effects.

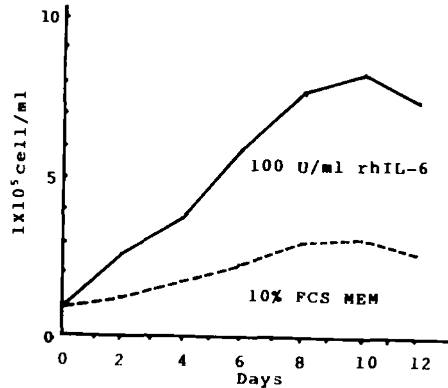

Fig 3. Analysis of the effect of exogenous recombinant human IL-6 (100U/ml) on in vitro growth of medulloblastoma cells. Med-3 cells incubated in normal culture medium were used as control.

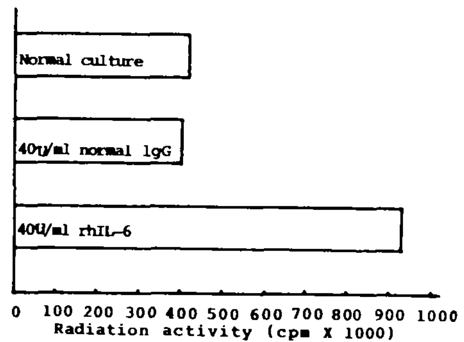

Fig 4. The influence of exogenous recombinant human IL-6 (40U/ml) in activity of DNA synthesis of Med-3 cells. Med-3 cells incubated in normal culture medium and in the medium containing 40U/ml normal IgG were used as normnal and nonspecific control, respectively.

DISCUSSION

Most studies concerning IL-6 genes in human malignancies were performed on hematopoietic cell,[14-16] and revealed that it could act as inducer or inhibitor

of growth and differentiation, which may largely depend upon the type/stage of cell lineages. Medulloblastomas are believed to originate from neuroectodermal stem cells during their maturation, resulting in one or several partially differentiated cell population(s) with an unlimited proliferation status.[1] We, therefore, hypothesized that, if constitutive IL-6 expression has such effects on malignant hematopoietic cells, it might also play certain role(s) in proliferation/differentiation of medulloblastomas. To address this issue, we checked *in vivo* and *in vitro* expression of IL-6 and its receptor genes in medulloblastoma cells by different methods. A high incidence (>90%) of IL-6R or gp 130 gene expression could be found in medulloblastoma tumors and cell line, but none of those samples expressed IL-6 either at the gene or the protein levels. Since the tumor samples we studied were obtained from both male and female patients with different ages and differentiation lineages, our data may indicate 1) that the absence of IL-6 expression may be a general feature of medulloblatomas and 2) that IL-6, though absent, can still exert its effect paracrinally on medulloblastoma cells because of the existence of IL-6R and gp 130 in the cells.

An established medulloblastoma cell line, Med-3 was then used in this study to assess the functional role of IL-6 on medulloblastoma cells. It was found that IL-6 could significantly stimulate both proliferation and DNA synthetic activity of Med-3 cells. These results were reasonable, because the target cells, though not expressing IL-6 by themselves, keep a complete set of signal responding system, gp 130 and IL-6R, for IL-6. Since medulloblastomas, as well as other primary brain tumors, are surrounded by gliosis and infiltrated by immune activated cells which have been known to express IL-6,[7] the influence of IL-6 released from those cells may also contribute to the proliferation of the tumors. Therefore, the current data may have potential clinical significance.

It has been known that IL-6 shares similar biological functions with several factors, such as leukemia inhibitory factor (LIF), IL-11, oncostatin M and CNTF.[16-18] Since those factors are usually co-expressed in many cell systems and, as a result, form a powerful functional compensation facility/network, the loss of one participant, e.g. IL-6, can be redressed by other factors with analogous functions like LIF, oncostatin M (OSM), CNTF and IL-11. This capacity of swap functions have produced a dilemma in cancer therapy oriented to those growth factors. However, the results of present study provide, for the first, an unique cell syustem in which IL-6 is totally absent but its growth stimulating effect may be compensated by certain factor(s) with IL-6 like bioactivities within medulloblastoma cells. Therefore, it is worthwhile to further characterize the expression and production of IL-6 like growth factors in meduloblastomas and to analyse cellular changes when the transcription and/or signal transduction of those factor(s) is blocked.

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