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원 저

Microarray analysis of hypoxia-induced changes in gene expression in BV-2 microglial cells

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국문초록

BV-2 microglia 세포주에서 저산소증의 유전자 발현에 대한 마이크로어레이 분석

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목적 : 허혈시 발생되는 저산소증 상태에서는 세포독성을 유발한다고 알려져 있으나 정확한 기전은 아직 규명되지 않았다. 본 연구에서는 뇌허혈로 인한 세포독성의 기전을 유전자 발현을 통하여 살펴보고자 하였다.

방법 : 본 실험에서는 BV-2 microglia 세포주에 12시간 동안의 저산소 상태에서의 유전자 발현을 분석하기 위하여 마이크로에레이를 시행하였다.

결과 : 저산소 상태에서는 정상에 비하여 cathepsin F, growth factor independent 1, calcitonin/calcitonin-related poly, leucine-rich repeat LGI family membrane, dublecortin, cyclohydrolase 1, Ia-associated invariant chain, carbohydrate kinase-like과 erythrocyte protein band 4.1-like 3 등의 유전자 발현이 3배 이상 증가하였다. 한편 neuronal guanine nucleotide exchange factor, Bcl-2-related ovarian killer protein, chemokine(C-X-C motif) ligand 5, RNA binding motif protein 3, interleukin 2 receptor, alpha chain, crystallin zeta, cytochrome P450 subfamily IV B, asparagine synthetase과 moesin 등의 유전자 발현은 0.2배 이하로 감소하였다.

결론 : 이상의 결과는 저산소증에 관여하는 유전자 및 저산소증과 관련된 뇌경색 등의 질환의 기전을 밝히는데 기초적 자료로 이용될 수 있을 것이다.

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I. Introduction

Hypoxia is critical physiological and pathological stimulus in a variety of disease states and has dual characters, one is beneficial, the other is harmful[1)~3)]. In the brain, hypoxia might act as preconditioning events and also cause significant damage in extended periods[4)]. Recently mechanisms by which cells respond to hypoxia have begun to be elucidated[5)].

Brain is a most susceptible organ to hypoxia[3)]. The brain may be exposed to hypoxia under a number of conditions such as severe hemorrhage, cerebral ischemia, high altitude, and anemia. The extent and duration of hypoxia, in addition to the presence of other confounding factors such as tissue ischemia, will determine the brain response to diminished oxygen supply. Hypoxia alters the brain protein patterns through the altered gene expression, posttranscriptional modification, and protein degradation. It is also known that hypoxia generally induces several transcription factors such as HIF-1 and NF-êB[6)] and several target genes[7)].

Several transcription factors such as, HIF-1(hypoxia-inducible factor-1, AP-1(activating protein-1), NF-kB(nuclear factor kB), and HSF-1(heat shock factor-1) and genes involving cell growth, intracellular metabolism and signaling are known to be induced by hypoxia[8)]. For example, HIF-1 increases expression of many genes, including VEGF(vascular endothelial growth factor), various enzymes associated with gluconeogenesis and glycolysis[9),10)]. Considering the many transcription factors and genes are influenced by hypoxia, hypoxia itself may exert a switch of many genes. Thus, several studies are focused on the identifying the genes, which are changed by hypoxia and a growing number of hypoxia-responsive genes have now been identified[10),11)]. But hypoxia is a whole cell event and because almost genes are responded to hypoxia, these researches have limitations. With DNA microarray technology and the availability of mouse genome, gene expression patterns and changes in genomic responses to the various stimuli can be detected. With this technology, shortcomings in studying the altered gene expression in the many pathologic states are resolved. We used mouse cDNA chip by Digital Genomics to investigate the hypoxia-induced changes in gene expression in BV2 microglial cells. Genes, which are selected by mouse genome were tested according to manufacturer's instruction. Using TwinChip™ Mouse, we screened the changes in the 5592 mouse genes in BV-2 microglial cells between control and hypoxia group.

Ⅱ. Materials and methods

1. Cell culture and hypoxia treatment

Mouse microglia cell line BV-2 was purchased by ATCC(Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagles medium(DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum(Gibco BRL) at 37℃ in 5% CO_2, 95% air in humidified cell incubator. To induce hypoxia, the cells were incubated in dessicators containing water and BBL GasPak Pouch System(Becton-Dickinson, Sparks, MD, USA). This system provides a compact microenvironment contained in an impermeable bag (dessicators) that provides anaerobic conditions with an O_2 concentration of <2% within 2 hr of incubation at 37℃. The catalytic reaction reaction that ensues consumes O_2 and produces CO_2[12].

2. RNA extraction

After 12 hr of hypoxia, cells were harvested and total RNA was isolated with TRIzol reagent (Gibco BRL) according to the manufacturer's protocol. Briefly, 1ml of TRIzol was added to the cells($1x10^7$), and the lysate was then extracted with chloroform and precipitated by isopropyl alcohol and subjected to further purification using an RNeasy mini kit(Qiagen, Valencia, CA, USA). The quality and quantity of extracted total RNA samples were examined by loading 10㎍ of samples on a denaturing agarose gel.

3. Microarray analysis

Array expression analysis was performed using the TwinChip™ Mouse-6K array(Digital Genomics, Seoul, Korea).

Double stranded cDN A is synthesized from total RNA. The Digital Genomics TwinChip™ Mouse-6K array an- alyses 5592 full-length sequences(3377 known genes and 2215 unknown EST clusters).

For microarray analysis, cDNA probes were generated by standard reverse transcriptase reaction in which some of the dTTP was replaced with either 50 uM Cy3-labeled dUTP or 75 uM Cy5-labeled dUTP(Amershan Pharmacia Biotech) using 10㎍ RNA from control and hypoxia group. Probes were cleaned using the QIAquick nucleotide removal kit(QIAGEN Inc.).

Probes were combined and hybridized to the array overnight at 58? in buffer containing 0.57 ㎍/㎕ COT-1 DNA, 0.57㎍/㎕(dA)40~60, 0.23 ㎍/㎕ yeast RNA, 3.5xSSC, and 0.3% SDS. Slides were washed in the following buffers at room temperature : 1) 10min in 2xSSC and 0.2% SDS ; 2) 5min in 1xSSC and 0.2% SSC ; 3) 1min in 2xSSC ; 4) 1min in 0.05xSSC. Slides were then dried by centrifugation at room temperature and scanned.

Slides were scanned with a Scanarry scanner(Packard Inc.) at 532 nm(Cy3) and 635(Cy 5) simultaneously. The images were analyzed using GenePix software(Axon Inc.). The background-subtracted median ratio value was calculated for each spot, and replicate spots on each slide were averaged.

Ⅲ. Results

1. Up-regulated genes

Treatment of cells for 12hr in hypoxic condition increased the expression of 13 genes. The list of genes include cathepsin F, growth factor independent 1, calcitonin/calcitonin-related poly, leucine-rich repeat LGI family membrane, dublecortin, cyclohydrolase 1, Ia-associated invariant chain, carbohydrate kinase-like, erythrocyte protein band 4.1-like 3 and 3 EST sequences<Table 1, Figure 1>.

Table 1. Genes up-regulated in BV2 microglial cells after 12hr hypoxia

Gene Name	Induction Fold
1200014I03Rik, RIKEN cDNA 1200014I03 gene	6.36
Ctsf, cathepsin F	6.17
Gfi1, growth factor independent 1	5.61
Calca, calcitonin/calcitonin-related poly	3.63
Es2el, expressed sequence 2 embryonic lethal	3.58
Lgi3, leucine-rich repeat LGI family, membrane	3.40
Dcx, doublecortin	3.34
Gch, GTP cyclohydrolase 1	3.30
2900074L19Rik, RIKEN cDNA 2900074L19 gene	3.30
Ii,Ia-associated invariant chain	3.21
Carkl, carbohydrate kinase-like	3.21
9030425C21Rik, RIKEN cDNA 9030425C21 gene	3.18
Epb4.1l3, erythrocyte protein band 4.1-like 3	3.01

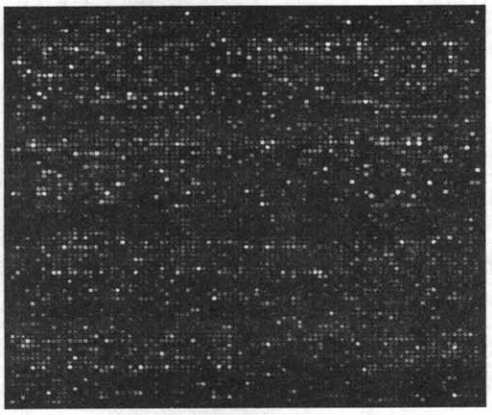

Figure 1. Cyanine dye-labelled mouse BV-2 microglia cell cDNA was hybridized to the microarray.

A two color image of a BV-2 microglia cell cDNA microarray is shown, red dots represent Cy3, and green dots represent Cy5. After scanning for each fluorescent dye false color images were superimposed. Red dots and green dots represent genes that show altered expression, and yellow dots represent genes that show no change in expressioin.

2. Down-regulated genes

Treatment of cells for 12hr in hypoxic condition decreased the expression of 12 genes. The list of genes include neuronal guanine nucleotide exchange factor, Bcl-2-related ovarian killer protein, chemokine(C-X-C motif) ligand 5, RNA binding motif protein 3, interleukin 2 receptor, alpha chain, crystallin zeta, cytochrome P450 subfamily IV B, asparagine synthetase, moesin and 3 EST sequences <Table 2, Figure 1>.

Table 2. Genes down-regulated in BV2 microglial cells after 12 hr hypoxia

Gene Name	Induction Fold
Ngef, neuronal guanine nucleotide exchange factor	0.21
Bok, Bcl-2-related ovarian killer protein	0.21
ESTs, Weakly similar to RIKEN cDNA 9	0.18
AI465173, expressed sequence AI465173	0.22
Cxcl5, chemokine(C-X-C motif) ligand 5	0.28
Rbm3, RNA binding motif protein 3	0.25
Il2ra, interleukin 2 receptor, alpha chain	0.29
ESTs, Weakly similar to 2208398A disi	0.21
Cryz, crystallin, zeta	0.23
Cyp4b1, cytochrome P450, subfamily IV B,	0.27
Asns, asparagine synthetase	0.29
Msn, moesin	0.27

Ⅳ. Discussion and Conclusion

Hypoxia is a critical pathophysiological stimulus in a variety of diseases and is a major cause of various neurologic diseases. Although hypoxia generally suppresses brain function, it also has also preconditioning effect[4]. The response of the brain to hypoxic stimuli is regulated mainly by changing the gene expressions. Over the decade, many genes involved in hypoxia have been identified individually or systemically. But these studies have identified only limited number of genes. But microarray analysis offered new insight to the molecular basis of brain's response to hypoxia. The limitations of the present study are common to all microarray analysis. First, technically some of the microarray data may be wrong. Second, the changed transcription does not always imply increased translation. Although microarray analysis has these limitations, this study is very effective in screening large number of genes.

Many studies are focused to identify the genes, which involve in hypoxia-induced changes in nerve cells, heart cells, and animals[10),11),13)]. Many genes, such as, for HIF (hypoxia-inducible factor), VEGF(vascular endothelial growth factor), c-fos, Bax, Bcl-2 are involved in hypoxia-induced changes[7),14),15)]. These genes can be classified as transcription factor, cytokines, growth factors, apoptosis genes, antioxidants genes, and genes associated with metabolism. But according to the time and conditions of hypoxia, genes expressed in hypoxic damage are differ[10),13)]. In our study, we identified 13 up-regulated genes and 12 down-regulated genes. Most of genes, which are changed in this study are new genes. The up-regulated gene are as bellows. Cathepsin is papain family cysteine proteases and is known to one of the apoptosis genes[16)]. Doublecortin(DCX) and erythrocyte membrane protein band 4.1 are involved in cell structure[13),17)]. Genes involved in metabolism are GTP cyclohydrolase 1(GCH1)

and carbohydrate kinase-like(CARK L)[18),19)]. CD74 antigen gene in immune system is also up-regulated[20),21)]. The down-regulated genes included neuronal guanine nucleotide exchange factor(NGEF)[22)], Bcl-2-related ovarian killer (BOK)[23)], chemokine CXC motif ligand 5(CXCL 5)[24)], RNA-binding motif protein 3(RBM3)[25)], interleukin 2 receptor alpha(IL2RA)[26)], crystalline zeta(CRYZ)[27)], cytochrome P450 subfamily IVB member 1(CYP4B1)[28)], asparagines synthetase(ASNS)[29)], and moesin(MSN)[30)]. These genes are not identified from other hypoxia-ishcemia studies.

Understanding the molecular mechanism involving hypoxia could offer good chances to reveal the mechanism of ischemic preconditioning and ischemic brain damage. The development of microarray systems for gene expression profiling permits screening of large numbers of genes for possible involvement in biological or pathological processes. Therefore, we used a cDNA microarray consisting of 5592 mouse genes in order to search new molecular targets of hypoxia responsive genes in microglia cells. And we found that several genes were changed in hypoxia in BV-2 mi- croglial cells. But these genes are not known yet to involve in hypoxic damage. Although further studies are need, there are possi- bilities one of these genes are involved in hypoxia-induced changes.

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