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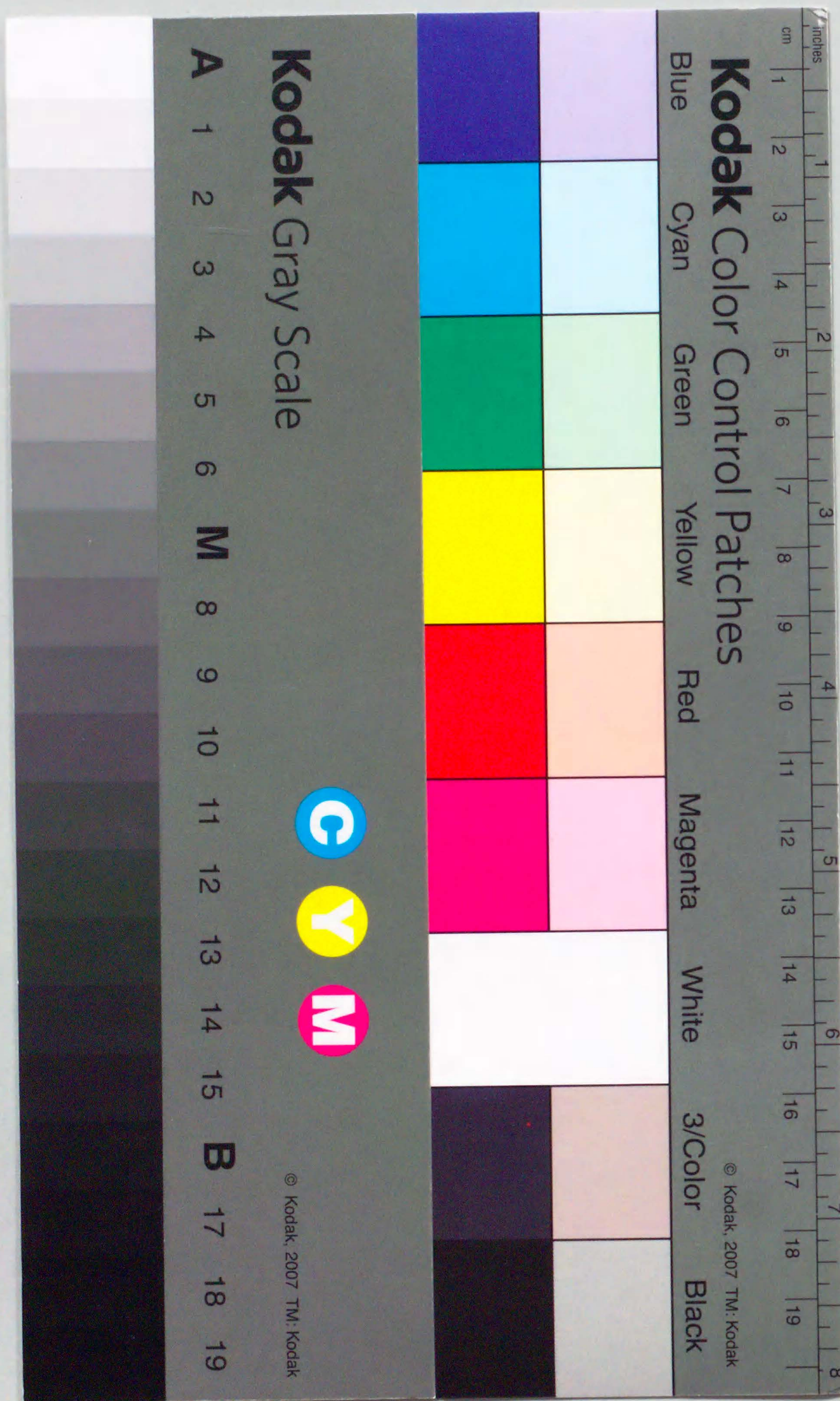

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BRAIN RESEARCH

Short Communication

Hemoglobin as a novel protein developmentally regulated in neurons

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Abstract

We have attempted to identify some novel genes which were found to be more highly expressed in the embryonic brain than in the adult brain. Consequently, one of these clones was identified as α-globin cDNA. Actually, α-globin mRNA was detected in the neurons. In addition, β-globin mRNA was detected in the neurons as well. Both globin mRNAs were developmentally regulated in the same pattern. Subsequently, further examinations with antiserum to hemoglobin revealed the presence of hemoglobin in the neurons. Hemoglobin has, up to now, been known to be an important O_2 transporter protein in erythrocytes. Moreover, hemoglobin is now considered to be also a very dangerous protein generating the toxic hydroxyl radical (·OH). We herein show the presence of hemoglobin and its regulation in the central nervous system, which may indicate the presence of a useful function regulating O_2 homeostasis and a potential oxidative toxicity for neuronal cells.

Key words: Hemoglobin; Neuron; Developmental regulation; Free radical; Neuronal degeneration

Although several factors and cytokines are known to contribute partly to neuronal maturation [19], it is still unclear as to how exactly neuronal development progresses during the embryonic period. To better understand this process, we attempted to identify any novel genes, which would regulate the development of the brain, by screening an embryonic mouse brain cDNA library. Since we guessed that genes essential for the neuronal maturation would be more expressed at an embryonic stage than at an adult stage in the brain, we carried out a differential hybridization (+/− method) similar to that of a previous report [5]. In that report, they have found out novel cDNAs which might be related to neuronal regeneration. Unexpectedly, in this report, we found out the hemoglobin as a novel protein developmentally regulated in neurons. Recent studies [3,9,27] have suggested the importance of iron metabolism for the normal brain function and for the cause of several neurodegenerative diseases. Therefore, the neuronal hemoglobin reported here may be a new important protein in the central nervous system.

We first constructed a cDNA library from BALB/c mouse embryonic day-17 (E17) brains using the cDNA synthesis kit (Pharmacia) and screened it with the differential cDNA probes. As a matter of fact, the colonies of library were transfered to a couple of

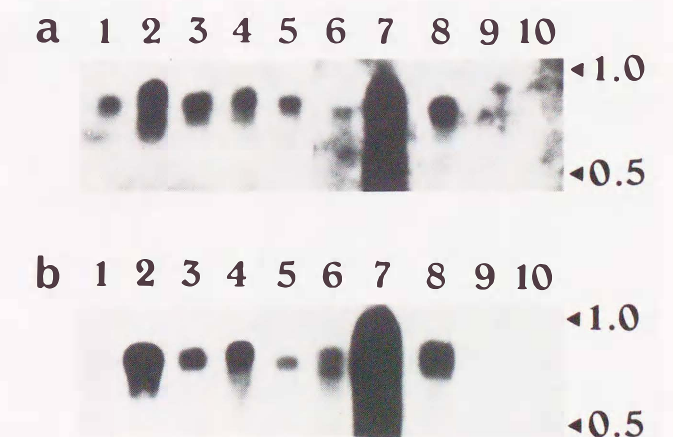

Fig. 1. Northern blot analysis of α-globin (a) and β-globin (b) mRNAs expression in the tissues and primary cultures. Lane 1, Embryonic day-13 (E13) brain; lane 2, E17 brain; lane 3, postnatal day-0 (P0) brain; lane 4, P5 brain; lane 5, adult (about 4 months old) brain; lane 6, adult liver; lane 7, E17 liver; lane 8, primary neurons cultured for 15 h after dissociation; lane 9, primary neurons cultured for 63 h after dissociation; lane 10, primary glial cells cultured for 3 days. The markers on the right side are represented in kilobases (kb). Each 20 μg of total RNA was analyzed.

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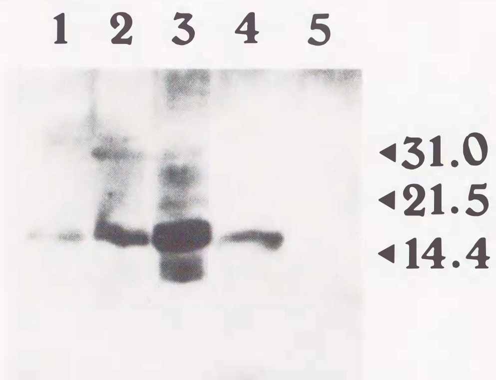

Fig. 2. Western blot analysis of globins in the tissues and primary cultures. Lane 1, E17 brain; lane 2, adult brain; lane 3, E17 liver; lane 4, primary neurons cultured for 15 h; lane 5, primary glial cells cultured for 3 days. The markers on the right side are represented in kilodaltons (kDa). Each 40 μg (lane 1–3) or 20 μg (lane 4, 5) of total protein was analyzed.

Hybond-N + nylon membrane (Amersham) and grew up to the large size, followed by treatment with 0.5 M NaOH/1.5 M NaCl, neutralization and hybridization with the highly ^{32}P-labeled cDNA probes prepared from the E17 brain and the adult brain. After the autoradiography, a positive-negative selection was carried out. Out of 4000 clones, we obtained some clones whose signals were both positive for the E17 probe and negative for the adult one. The DNA sequencing analysis and homology search revealed that two of them were completely identical to the mouse α-globin cDNA. Hence we performed a northern blot analysis of α-globin, which method was previously described [17], to confirm its expression in brain.

As shown in Fig. 1a, the expression of α-globin mRNA was markedly increased in the late embryonic stage and gradually decreased after birth. Its very abundant expression was also found in an E17 liver undergoing extramedullary hemopoiesis. In order to rule out the possibility of contamination with the hemopoietic cells and clarify the cellular origins of α-globin in the brain, both primary neuronal and glial cultures were examined. The primary cultured neurons and glial cells were obtained from E15 and neonatal BALB/c mouse brain, respectively, according to previous reports [16,17]. Their populational purities have already been previously evaluated [12,16,17]. Additionally, in this study, immunocytochemical tests have also revealed that each cell culture consists of pure neurofilament (NF)-positive (neuron) or glial fibrillary acidic protein (GFAP)-positive (astrocyte) cells (Fig. 3e,h). A definite expression of α-globin was found in the neurons 15 h after dissociation, but disappeared during further cultivation for 48 h. The glial cultures dissimilarly showed no α-globin expression. These results thus indicated the existence of the neuronal α-globin expression at least at a certain stage. However, as the glial cells had to take several days to differentiate and proliferate in vitro, those cells cultured for 3 days were used for the examination. Thereby, the expression in immature glias are unsettled. The mechanism of α-globin gene repression in cultured neurons was unclear. Recently, De Leon et al. [5] reported that the α-globin expression in the peripheral nerve was markedly repressed when nerve regeneration occurs after injury, but they did not confirm the cellular origin. If it would be the neuronal origin, the α-globin mRNA might be repressed during neurite outgrowth in vivo as well as in vitro.

α-globin is ordinarily molded into hemoglobin jointly with non-α globin such as β-globin in erythrocytes ($\alpha_2\beta_2$) [23]. We next studied the expression patterns of β-globin mRNA in the same samples. Although the size of β-globin mRNA is similar to α-globin one, the β-globin gene was distinguishable from the α-globin one by a northern blot analysis because they demonstrated little homology. As shown in Fig. 1b, its expression patterns were quite similar to the α-globin ones. Moreover, a recent study have showed a marked expression of transferrin receptor and the uptake of transferrin, a major iron transporter, in embryonic neurons [15]. Since such iron could be converted into heme in the mitochondria [23], it may be rational to suppose that hemoglobins are vigorously synthesized in developing neurons.

We then performed a western blot analysis according to the manual [24] and immunocytochemical studies using an antiserum to mouse hemoglobin (Cappel, NC). Fig. 2 shows that common 15 kDa bands are detected with the antibody and these bands correspond to the monomer of the globin protein [6] in the E17 brain, the adult brain, the E17 liver and the cultured neurons, but not in the cultured glias. These findings all were similar to the results of the northern blot analysis. However, in contrast to the northern blot

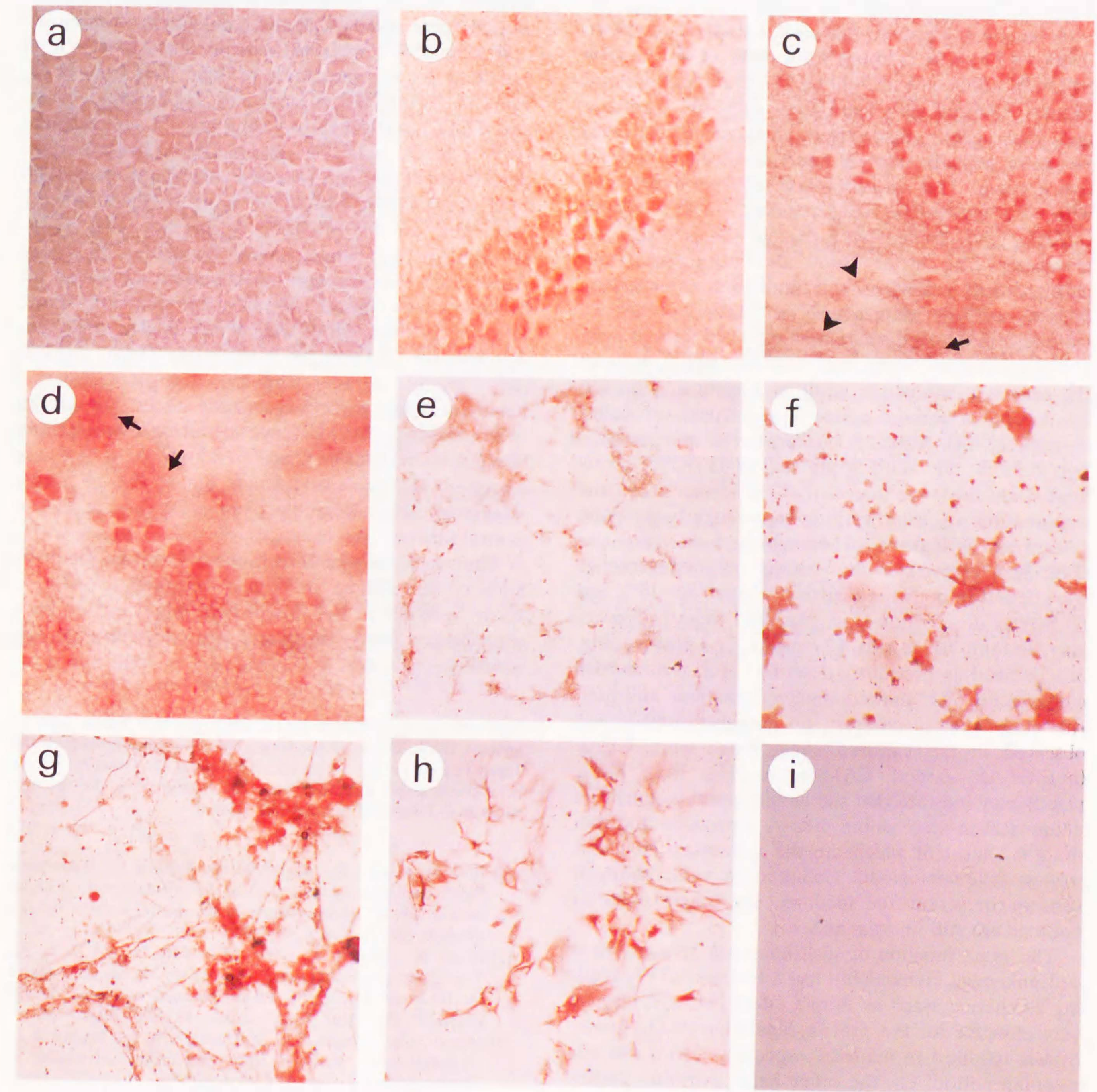

Fig. 3. The immunostainings of the mouse brain and primary cultures for hemoglobin (Hb), neurofilament (NF) and glial fibrillary acidic protein (GFAP). a: E17 brain tissue (Hb, 1:1000). b: adult hippo-campal (CA3) region (Hb, 1:1000). c: adult parietal cortical region (Hb, 1:1000). d: adult cerebellar cortical region (Purkinje and granule cells) (Hb, 1:1000). e: primary neurons cultured for 15 h (NF, 1:200). f: primary neurons cultured for 15 h (Hb, 1:1000). g: primary neurons cultured for 63 h (Hb, 1:1000). h: primary glial cells cultured for 3 days (GFAP, ready-to-use). i: primary glial cells cultured for 3 days (Hb, 1:1000). Magnification: a–c, ×200; d–i, ×100.

analysis, the amount of globin protein in the adult brain was slightly more than that in the E17 brain, which may reflect the contamination with hemoglobin of erythrocytes in the abundant vessels of the adult brain. All these bands included both globins, which was confirmed with the specific antibodies (Cosmobio, Tokyo) (data not shown).

Subsequent immunocytochemical studies are shown in Fig. 3. The mouse brains were removed and quickly frozen in isopentane at −70°C. The brain sections and primary cultured cells were fixed with 10% formalin and cold aceton, respectively. The specimens were incubated with the primary antibodies for 2 h at room temperature. The immunoreactivities were detected with the peroxidase-antiperoxidase (PAP) system (DAKO, CA). Aminoethyl carbazole was used as a red chromogen [26]. In this study, all control tissues and cultured cells lacking primary antibodies exhibited no immunoreactivity (data not shown). Consequently, we were able to detect definite immunoreactivities in most of the embryonic brain cells (Fig. 3a) and in most of the neuronal cells of the adult hippocampus (Fig. 3b), basal ganglia, parietal cortex (Fig. 3c) and cerebellar cortex (Fig. 3d). Although the number of immunoreactive cells in the adult white matter (Fig. 3c, arrow heads) was far lower than that in the former areas, the examination was insufficient owing to high background caused by the leakage of hemoglobin from capillaries (Fig. 3c,d, arrows). While, strong immunoreactivities were found in both the neurons cultured for 15 h and 63 h after dissociation (Fig. 3f,g) and these cells were also NF (anti-NF 68 kDa, Chemicon, CA)-positive (Fig. 3e). As well as previously described [16], the neurons cultured for 63 h showed cluster formations and neurite outgrowth. In contrast, no immunoreactivity was observed in the cultured glias, which were GFAP (anti-GFAP, Zymed, CA)-positive (Fig. 3h,i). These results may indicate that the globin proteins remain in neurons even after globin mRNA repression and that the glias, most of which are the astrocytes, may thus possess little hemoglobin. Owing to the vulnerability of neurons in serum-free medium, the longer cultured neurons are still unexamined.

The exact function of such neuronal hemoglobin is still unknown. Hemoglobin has a function of O_2 binding as characterized as Bohr's effect [23] and may be very effective for the precise regulation of O_2 homeostasis required in neurons, especially during the developing period. On the other hand, previous studies have shown that oligodendrocytes make transferrin (for iron mobilization) in the rat brain [2,7] and that microglias [13] and other glial cells [4] possess ferritin (for iron storage). Furthermore, a recent report has shown the presence of hemopexin, which is the heme-recycling protein, in the human neurons [14]. The neuronal hemoglobin (as an iron consumer) reported herein may also be involved in the iron metabolism and would be regulated in harmony with such iron-regulatory proteins in the brain.

It is increasingly of interest to note that free radicals such as the hydroxyl radical ($\cdot OH$) may contribute to some neurodegenerative diseases such as Parkinson's disease [11,27], Alzheimer's disease [25], Hallervorden-Spatz disease [20] and familial amyotrophic lateral sclerosis [22]. Hemoglobin is thus an important candidate for the generation of toxic $\cdot OH$ from H_2O_2 when the hemes are degraded by, for example, excessive H_2O_2 and free iron ions (Fe^{2+}) are released ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$, Fenton reaction) [9,10]. Free iron ions also contribute to cellular lipid peroxidation related to aging in the brain [1]. Moreover, certain insoluble formations such as β-amyloid [6] and paired helical filaments [28] in the Alzheimer's disease brain might also be accounted for by free radicals. Indeed, free irons are frequently found in senile plaques [3], as well as the degenerated substantia nigra [27] and the degenerated globus pallidus [18]. While, it now appears that the extrinsic hemoglobin has a great neurotoxic effect through the $\cdot OH$ generation [8,21]. Hence the intrinsic hemoglobin of neurons can thus be considered not only to be a very important O_2-regulatory protein but to also be a very dangerous and potentially pathogenic protein in the central nervous system.

Therefore, we should continue to examine the presence of hemoglobin in human neurons in order to clarify whether or not this protein might indeed be a contributory factor to several neurodegenerative diseases.

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