TABLE 1

*Clinical characteristics of three groups of severely head injured patients**

Characteristic	Extracerebral Hematoma Group	Focal Cerebral Lesion Group	Diffuse Swelling Group
no. of patients	8	6	8
M/F ratio	6:2	2:4	4:4
mean age (yrs)	53 ± 18	47 ± 21	39 ± 24
pupil abnormalities on admission (%)†	7 (87.5)	3 (50.0)	8 (100)
hypoxia on admission (%)‡	6 (75.0)	4 (66.7)	4 (50.0)
hypotension on admission (%)§	1 (12.5)	2 (33.3)	2 (25.0)
mean ICP before inducing moderate hypothermia	55 ± 14	43 ± 4	54 ± 9

* There was no significant difference among groups. Values are expressed as the means ± standard deviation.

† Pupil abnormalities were defined as abnormalities in size and/or reaction to light in one or both pupils.

‡ Hypoxia was defined as PaO_2 less than 60 mm Hg.

§ Hypotension was defined as a sustained fall in systolic blood pressure to 100 mm Hg.

Patients younger than 10 years were excluded from this study. In 60 patients, ICP was maintained below 20 mm Hg by using conventional therapies such as ventricular CSF drainage, mild hyperventilation, and/or high-dose barbiturate medications; these patients were excluded from this study. In 17 patients, ICP was regulated below 40 mm Hg by the use of mild hypothermia combined with conventional therapies; these patients were also excluded from the study. Fourteen additional patients were excluded because their ICP equaled their MABP before this study was initiated. Eleven patients with severe, life-threatening injuries to another organ were also excluded. Thus, only the remaining 22 patients whose ICP could not be maintained below 40 mm Hg with the use of mild hypothermia were included in this study. These patients (12 men and 10 women) ranged in age from 16 to 72 years, with a mean age of 46 years. Of the 22 patients, 18 were transported to our hospital directly from the scene of the accident within 40 minutes postinjury. The remaining four patients were referred from other hospitals within 2 hours after injury. Injuries resulted from a traffic accident in 17 patients, a fall in four, and an assault in one. The Glasgow Coma Scale[16] scores in all patients were 8 or less on admission. In each case, informed consent to participate in this study was obtained from the patient's family.

Patient Treatment

All patients were initially intubated, underwent continuous hyperventilation therapy with $PaCO_2$ between 30 and 35 mm Hg, and were treated with fluid resuscitation at 1 to 2 ml/kg/hr. We used CPP (MABP − ICP), central venous pressure, and urine output to determine the volume of the intravenous infusion and the dose of the dopamine infusion. If the CPP was less than 60 mm Hg, adequate amounts of albumin and/or continuous infusions of dopamine were given during the study period as needed. To maintain central venous pressure between 1 and 7 mm Hg, an adequate amount of colloid fluids was administered during the study period as needed. To maintain a urine output of greater than 0.5 ml/kg/hr, adequate amounts of colloid fluids and/or continuous infusions of dopamine were given during the study period as needed. No corticosteroid medication or mannitol was administered during the study. After initial resuscitation, all patients immediately underwent CT scanning of the head. Subsequently, an intraventricular catheter was inserted in each patient for continuous monitoring of ICP and intracranial temperature monitoring. If necessary, intracranial hematomas and/or hemorrhagic contusions associated with a midline displacement of more than 5 mm were evacuated intraoperatively.

In all patients, intracranial hypertension was initially managed using conventional ICP reduction therapies such as ventricular CSF drainage, mild hyperventilation, and/or high-dose barbiturate medications. Barbiturate therapy was administered according to a published protocol.[13,15] If ICP remained higher than 20 mm Hg after the administration of high-dose barbiturate medications, we induced mild hypothermia.

Mild hypothermia was induced as quickly as possible by placing cooling blankets above and below the patient and by performing nasogastric lavage with iced saline. The intracranial temperature, measured in the lateral ventricle, was maintained at 33.5 to 34.5°C. If we could not maintain ICP below 40 mm Hg by inducing mild hypothermia, we subsequently induced moderate hypothermia as quickly as possible by using the aforementioned method. Intracranial temperature was kept between 30.5 and 31.5°C.

As previously reported,[15] the efficacy of mild hypothermia was clearly related to the type of intracranial lesion demonstrated on CT studies: 1) the extracerebral hematoma group, composed of patients whose primary mass lesion was a subdural and/or an epidural hematoma; 2) the focal cerebral lesion group, composed of patients whose primary mass lesion was a brain contusion and/or an intracerebral hematoma; and 3) the diffuse swelling group, composed of patients who had compressed or obliterated basal cisterns and/or third ventricle without a significant intracranial mass lesion. Therefore, we assessed the effectiveness of moderate hypothermia on ICP according to these classifications.

Obtained Measurements

Mean arterial blood pressure, heart rate, and ICP were continuously measured automatically. Serum concentrations of Na and K, WBC counts, and platelet counts were determined at 34 and 31°C in our hospital laboratory. The base excess in arterial blood was also measured at 34 and 31°C with the aid of a blood gas analyzer.

Statistical Analysis

All values are expressed as the means ± standard deviation. Changes in physiological measurements between 34 and 31°C were analyzed using the Wilcoxon matched-pairs test. Physiological measurements within groups were analyzed using one-way analysis of variance followed by the Scheffe test. Significance was assigned when the probability value was less than 0.05.

Results

Patient Characteristics

Of the 22 patients in the study, eight constituted the extracerebral hematoma group (all with acute subdural hema-

toma); six, the focal cerebral lesion group; and eight, the diffuse swelling group. Clinical characteristics in the three groups are summarized in Table 1. The mean time from hospital admission to achievement of the target temperature of 34°C did not differ significantly among the three groups: 4 ± 2.3 hours in the extracerebral hematoma group; 15 ± 28 hours in the focal cerebral lesion group; and 2.9± 1.4 hours in the diffuse swelling group. Given that the efficacy of mild hypothermia differed among the three groups,[15] the mean time from establishing the target temperature of 34°C to the time when ICP was greater than 40 mm Hg despite the use of mild hypothermia also differed significantly: 2.5 ± 3.3 hours in the extracerebral hematoma group; 28.7 ± 4.9 hours in the focal cerebral lesion group; and 1.6 ± 2.6 hours in the diffuse swelling group ($p < 0.01$). The mean time from the start of cooling to realizing the target temperature of 31°C did not differ significantly among the three groups: 3.1 ± 1 hours in the extracerebral hematoma group; 3.2 ± 0.8 hours in the focal cerebral lesion group; and 2.5 ± 0.8 hours in the diffuse swelling group. The brain temperature in patients in the extracerebral hematoma group reached 31°C at 9.6 ± 4.1 hours after admission; in patients in the focal cerebral lesion group, 46.8 ± 26.6 hours after admission; and in those in the diffuse swelling group, 6.9 ± 3.8 hours after admission ($p < 0.01$).

Changes in Physiological Measurements

As brain temperature was reduced from 34 to 31°C, the volume of intravenous fluid infusion was increased significantly from 1.9 ± 0.9 to 2.6 ± 1.2 mg/kg/hr ($p < 0.01$) and the dose of dopamine infusion was increased significantly from 4.3 ± 3.1 to 8.2 ± 4.4 μg/kg/min ($p < 0.01$). On the contrary, MABP and heart rate decreased significantly from 97.1 ± 13.1 at 34°C to 85.1 ± 10.5 mm Hg at 31°C ($p < 0.01$) and from 92.2 ± 13.8 at 34°C to 72.2 ± 14.3 beats/minute at 31°C ($p < 0.01$). Even worse, the arterial base excess was significantly aggravated from −3.3 ± 4 at 34°C to −5.6 ± 5.4 mEq/L at 31°C ($p < 0.05$). These results clearly demonstrate that, compared with mild hypothermia, moderate hypothermia provokes more severe cardiosuppression and more insufficient systemic circulation.

Serum concentrations of K decreased significantly from 3.1 ± 0.6 at 34°C to 2.5 ± 0.5 mEq/L at 31°C, even though an adequate amount of K had been administered ($p < 0.01$). Serious cardiac arrhythmia with cardiovascular instability was not observed during the study period. With respect to serum concentrations of Na, there was no significant difference between 139.6 ± 5.7 at 34°C and 141.3 ± 6.2 mEq/L at 31°C.

The WBC counts decreased significantly from 15.2 ± 5.3 × 10^9/L at 34°C to 11.9 ± 4.7 × 10^9/L at 31°C ($p < 0.01$). We found no correlation between the decrease in WBC counts and the incidence of complications of infection.

Platelet counts decreased significantly from a value of 159 ± 78 × 10^9/L at 34°C to 105 ± 62 × 10^9/L at 31°C ($p < 0.01$). No clinically important hypothermia-induced coagulopathy or bleeding was observed in the present study.

Effectiveness of Moderate Hypothermia on ICP According to CT Findings

Serial changes in ICP with the induction of moderate hypothermia are shown in Fig. 1. As with mild hypothermia, the efficacy of moderate hypothermia was clearly related to the type of intracranial lesion demonstrated on CT findings.

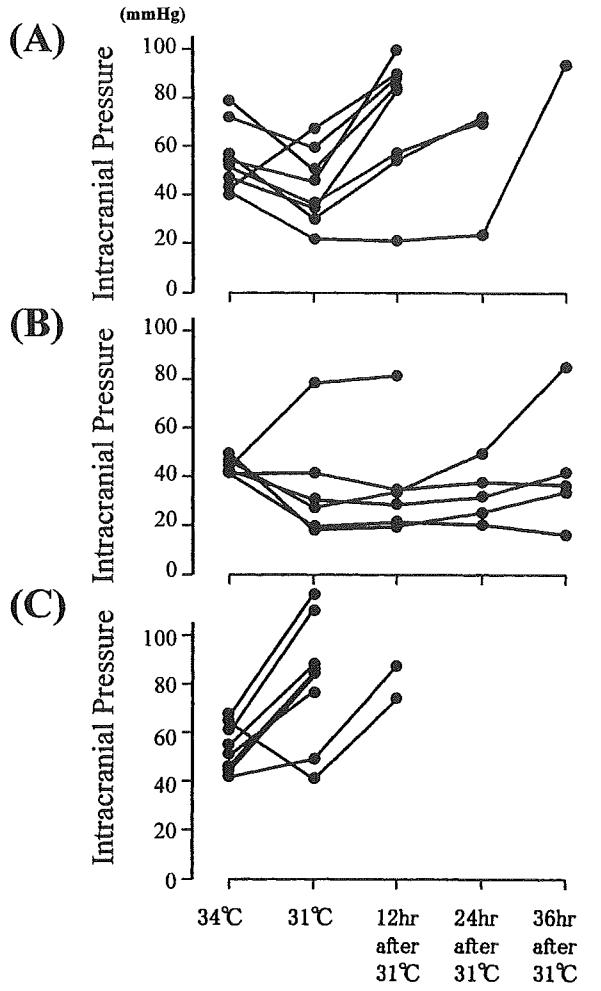

FIG. 1. Graphs depicting serial changes in ICP for individual patients on induction of moderate hypothermia (31°C). A: Extracerebral hematoma group (eight patients). B: Focal cerebral lesion group (six patients). C: Diffuse swelling group (eight patients).

In patients in the extracerebral hematoma group (Fig. 1A), moderate hypothermia did not prevent a rise in ICP. Seven of eight patients in this group died within 48 hours after injury as a result of refractory intracranial hypertension, and the remaining patient died within 72 hours after injury of the same cause.

We previously reported that patients with focal lesions responded quite well to mild hypothermia.[15] In the current study, the induction of moderate hypothermia successfully prevented progressive elevation of ICP in three of the six patients in the focal cerebral lesion group (Fig. 1B). Note, however, that the prevention of an increase in ICP with moderate hypothermia, unlike with mild hypothermia, was unrelated to improved clinical outcome. Three patients died of multiple organ failure: two on the 4th day and one on the 8th day in the hospital. The remaining three patients died within 1 week after injury as a result of refractory intracranial hypertension: one on the 3rd day in the hospital, one on the 5th day, and one on the 7th day.

As shown in Fig. 1C, the effect of moderate hypothermia on high ICP in patients in the diffuse swelling group was the worst among the three groups. Like the lack of response recorded with the induction of mild hypothermia, patients with diffuse swelling did not respond at all to moderate hypothermia. All eight patients died of refractory intracranial hypertension within 48 hours after injury.

Discussion

This prospective study was designed to answer the following two questions. 1) Can moderate hypothermia prevent the elevation of ICP and improve clinical outcome in severely head injured patients whose intracranial hypertension cannot be controlled with the use of mild hypothermia? 2) Does moderate hypothermia provoke complications more severe than those caused by mild hypothermia?

In the present study, we clearly demonstrated that moderate hypothermia was useless in preventing the elevation of ICP in patients with fatal intracranial hypertension. Although the mechanistic details are still being debated, a reduction in ICP induction by mild-to-moderate hypothermia has been generally considered to result from the following two factors: a decrease in CBF[14] and a reduction of brain edema.[6,11] When CBF–$CMRO_2$ coupling reactivity is intact in brain vessels, a decrease in CBF seems to be occurring. If this reactivity is intact, the reduction in cerebral metabolism causes a decrease in both CBF and cerebral blood volume, which in turn leads to a drop in ICP.[17] Therefore, we hypothesized that the reactivity of brain vessels might be abolished or highly impaired in patients whose intracranial hypertension cannot be controlled using mild hypothermia. If this is true, it is logical that a further decrease in temperature would not prevent an elevation in ICP. The finding in our study that moderate hypothermia did not prevent the elevation of ICP in 19 (86%) of 22 patients strongly supports our hypothesis. In such patients, we believe that even the deepest hypothermia cannot prevent any amount of elevation in ICP.

A reduction in brain edema cannot be objectively assessed in the clinical setting. It is known that brain edema is associated with blood–brain barrier disruption.[1] Data from laboratory studies have revealed that moderate hypothermia reduces blood–brain barrier disruption following brain injury[6] and following cerebral ischemia.[4] It is thought that protection of the blood–brain barrier causes a reduction in brain edema, which may lead to a reduction in ICP.[6,10,11] We do not know whether the magnitude of the reduction depends on the depth of hypothermia. We believe, however, that the efficacy of a reduction in brain edema does not differ significantly between 31 and 34°C in the clinical setting, because moderate hypothermia did not prevent the elevation of ICP in patients whose ICP could not be controlled using mild hypothermia.

Data from numerous clinical studies of accidental decreases in body temperature have revealed that hypothermia affects every organ system and that its toxicity is increased as the depth of hypothermia increases.[2,11] In the current study, the volume of intravenous fluid infusion and the dose of dopamine infusion increased significantly during moderate hypothermia from those observed during mild hypothermia ($p < 0.01$). Nevertheless, MABP and heart rate decreased significantly during moderate hypothermia ($p < 0.01$). The arterial base excess was significantly aggravated during moderate hypothermia compared with that during mild hypothermia ($p < 0.05$). Moreover, serum K concentrations, WBC counts, and platelet counts at 31°C decreased significantly compared with those at 34°C ($p < 0.01$). Our results are in accord with those obtained by many other investigators.[2,5,9,11] In three of 22 patients in the present study, moderate hypothermia controlled fatal intracranial hypertension. Nonetheless, these three patients died of multiple organ failure within 8 days after injury, indicating indirectly that moderate hypothermia could provoke complications more severe than those induced by mild hypothermia.

In a previous study, we clearly demonstrated that mild hypothermia itself increased the risk of systemic complications.[5,12,13] In the present study, as was expected, moderate hypothermia provoked complications more severe than those provoked by mild hypothermia. Even so, if moderate hypothermia can improve clinical outcome in patients with fatal intracranial hypertension, its use might be justified. Note, however, that all 22 patients in the present study died within 8 days postinjury. In this study we do not address whether moderate hypothermia would have been more effective than mild hypothermia if inducted immediately. We cannot deny the possibility that the lack of efficacy of moderate hypothermia in our patients may be due to a truly refractory intracranial hypertension. To evaluate the benefits of both treatments appropriately, a prospective study should be undertaken to examine the efficacy of and complications related to the immediate induction of mild compared with moderate hypothermia in matching groups of patients. Regarding patients whose ICP cannot be controlled using mild hypothermia, we conclude that there is no need to induce moderate hypothermia.

Conclusions

We prospectively assessed the efficacy and safety of moderate hypothermia in the treatment of severely head injured patients whose ICP could not be controlled using mild hypothermia. Moderate hypothermia does not improve the clinical outcome in patients; 19 of our 22 patients died of refractory intracranial hypertension, and three died of multiple organ failure. Moreover, moderate hypothermia appears to cause systemic complications more severe than those prompted by mild hypothermia. Therefore, we conclude that there is no need to induce moderate hypothermia in severely head injured patients in whom ICP cannot be controlled with the aid of mild hypothermia.

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Address reprint requests to: Tadahiko Shiozaki, M.D., Ph.D., Department of Traumatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita-shi, Osaka 565-0871, Japan. email: shiozaki@hp-emerg.med.osaka-u.ac.jp.

Mild Hypothermia Reduces Expression of Heat Shock Protein 60 in Leukocytes from Severely Head-Injured Patients

Naoyuki Hashiguchi, MD, Tadahiko Shiozaki, MD, Hiroshi Ogura, MD, Hiroshi Tanaka, MD, Taichin Koh, BS, Mitsuhiro Noborio, MD, Keiko Fugita, BS, Pavel Akimau, MD, Yasuyuki Kuwagata, MD, Takeshi Shimazu, MD, and Hisashi Sugimoto, MD

Background: Infectious complications are among the most serious problems that occur in severely head-injured patients treated with mild hypothermia. The mechanism underlying the susceptibility to infection has not been clarified. Heat shock protein (HSP) 60 has been reported to play an essential role in innate immunity. Thus, we conducted a study to clarify the impact of mild hypothermia on the expression of HSPs in polymorphonuclear leukocytes (PMNLs) in severely head-injured patients.

Methods: Between September 1997 and November 1999, 17 severely head-injured patients with a Glasgow Coma Scale score of 8 or less at admission in whom intracranial pressure could be maintained below 20 mm Hg by conventional therapy were randomly assigned to two treatment groups: a mild hypothermia group (HT group, nine patients) and a normothermia group (NT group, eight patients). The HT group was subjected to mild hypothermia (intracranial temperature, 34°C) for 48 hours followed by rewarming at a rate of 1°C per day for 3 days, whereas the NT group was subjected to normothermia (intracranial temperature, 37°C) for 5 days. Blood samples were serially obtained at three time points; days 0 to 1, days 2 to 5, and days 6 to 14 after head injury. We measured the expression of HSP27, HSP60, HSP70, and HSP90 by flow cytometry.

Results: The two groups were similar with respect to prognostic factors, and there was no difference in clinical outcome. The expression of PMNL HSP60 in the HT group was significantly lower in all three time periods compared with that in the NT group ($p < 0.05$), whereas expression of the other HSPs did not differ significantly between the groups. The incidence of infectious complications was significantly increased in the HT group over that in the NT group ($p < 0.05$). In in vitro studies, PMNLs from 10 healthy volunteers were incubated at 37°C, 34°C, or 26°C for 1 hour with sodium arsenite (100 μmol/L), an HSP inducer. The expression of HSP60 at 26°C and 34°C was significantly lower than that at 37°C ($p < 0.05$), whereas expression of the other HSPs did not differ significantly at 26°C, 34°C, or 37°C.

Conclusion: Mild hypothermia reduces the expression of HSP60 in PMNLs from severely head-injured patients. Thus, mild hypothermia may suppress innate immunity.

Key Words: Heat shock protein 60, Head injury, Mild hypothermia, Infectious complication, Polymorphonuclear leukocyte, Flow cytometry.

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In 1993, we reported that mild hypothermia (34°C) was effective for controlling intracranial hypertension refractory to conventional therapies.[1] Accordingly, we have used mild hypothermia only after conventional therapies have failed to maintain intracranial pressure (ICP) below 20 mm Hg.[2,3] We later found in a multicenter, randomized, controlled trial that the incidence of infectious complications in patients treated with mild hypothermia was significantly higher than in patients subjected to normothermia (37°C), and we reported that mild hypothermia should not be used to treat severely head-injured patients in whom ICP can be maintained at less than 25 mm Hg by conventional therapies.[4] The precise mechanism of susceptibility to infection in patients treated with mild hypothermia has not been clarified, however.

Recently, we reported that the expression of heat shock proteins (HSPs) in polymorphonuclear leukocytes (PMNLs) from severe trauma[5] and severe septic patients[6] was markedly enhanced and that these HSPs may play an important role in regulating PMNL function. HSPs are a set of conserved proteins that confer tolerance to stress and play a major role in the pathophysiology of infection and inflammation. HSP60, in particular, has been reported to play an essential role in innate immunity.[7] HSP60 exists mainly in cell membranes, and it has been reported that gamma-delta T lymphocytes are activated by HSP60 in bacteria or in hosts lacking major histocompatibility-complex reactivity.[7,8] The objective of the present study was to measure HSP expression in PMNLs in severely head-injured patients and to clarify the impact of environmental

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From the Department of Traumatology and Critical Care Medicine, Tottori Prefectual Central Hospital (N.H., M.N.), Tottori, and Departments of Traumatology (H.O., Y.K., T.S., H.T., K.F., P.A., T.S., H.S.) and Clinical Investigation (T.K.), Osaka University Graduate School of Medicine, Osaka, Japan.
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Address for reprints: Naoyuki Hashiguchi, MD, Department of Traumatology (D-8), Osaka University Graduate School of Medicine, 2-15 Yamadaoka, Suita-shi, Osaka 565-0871, Japan; email: hashiguchi@mx51.tiki.ne.jp.

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Table 1 Clinical Characteristics of 17 Patients with Severe Head Injury Shown per Treatment Group*

Patient	Age (yr), Sex	GCS Score at Adimission	Pupil Abnormalities	CT Findings	ICP (mm Hg)†	Evacuated Mass‡	Additional Injuries	ISS Score	GOS Score§
Mild hypothermia (34°C) group									
1	27, M	4	+	Con, SAH	4	–	—	26	SvD
2	34, M	4	+	ICH, con, SAH	5	+	—	26	GR
3	18, M	4	+	SDH, con, SAH	17	+	Chest injury, arm and leg fracture	38	SvD
4	63, M	4	+	ICH, con	6	+	—	26	D
5	21, M	5	–	EDH, base Fx	14	+	—	26	MD
6	20, M	5	+	SDH, con, base Fx	12	–	Chest injury	35	MD
7	18, M	7	–	Con, SAH	13	–	Pelvic fracture	35	GR
8	20, M	8	+	SDH, base Fx	15	–	Chest injury, leg fracture	38	GR
9	40, M	8	+	EDH, SDH	14	+	—	26	GR
Mean ± SD	29.0 ± 14.9	5.4 ± 1.7			11.1 ± 4.8			30.7 ± 5.6	
Normothermia (34°C) group									
1	56, M	3	+	Con, SAH	14	–	—	26	MD
2	40, F	3	+	SDH, con, SAH, base Fx	5	+	Chest injury	35	MD
3	19, F	4	–	Con, base Fx	17	–	—	26	GR
4	42, M	5	+	Con, SAH	11	+	Chest injury, pelvic fracture	43	SvD
5	20, M	6	+	EDH, base Fx	8	+	—	26	GR
6	48, F	6	–	SDH, con	17	–	Chest injury, arm fracture	38	GR
7	48, M	8	+	SDH, base Fx	13	+	Chest injury	35	GR
8	40, M	8	–	Con, SAH, base Fx	10	–	Chest injury	35	GR
Mean ± SD	39.1 ± 13.2	5.4 ± 2.0			11.9 ± 4.2			33.0 ± 6.4	

Base Fx, skull base fracture; con, contusion; EDH, epidural hematoma; ICH, intracerebral hematoma; SAH, subarachnoid hemorrhage; SDH, subdural hematoma; ISS, Injury Severity Score; GR, good recovery; MD, moderate disability; SvD, severe disability; V, vegetative state; +, present; –, absent; SD, standard deviation; CT, computed tomographic.

* No statistical differences between the two groups.

† Intracranial pressure after conventional therapy.

‡ Mass surgically evacuated before randomization.

§ The GOS score 6 months after injury.

temperature on the expression of HSPs in PMNLs in clinical and in vitro studies.

MATERIALS AND METHODS

Patient Population

Between September 1997 and November 1999, a total of 44 severely head-injured patients who required continuous infusion of barbiturates to control intracranial hypertension were admitted to the Department of Traumatology at Osaka University Hospital. Patients younger than 10 years of age were excluded from the study. In 17 of the 44 patients, ICP equaled mean arterial blood pressure before study could be initiated. Five patients suffered severe life-threatening injury to another organ. Five additional patients required mild hypothermia (34°C) because we could not maintain their ICP below 20 mm Hg using conventional therapy such as mild hyperventilation and high-dose barbiturates. Thus, 17 patients in whom ICP was maintained below 20 mm Hg by conventional therapy were included in this study. These patients were divided randomly into two groups: a mild hypothermia group (HT group, nine patients) and a normothermia group (NT group, eight patients). The clinical characteristics of patients in the two groups are presented in Table 1. The Glasgow Coma Scale score for each patient at admission was 8 or less. For each patient, informed consent to participate in this study was obtained from a family member. Seventeen healthy volunteers (mean age, 31.7 ± 5.8 years) participated as control subjects.

Patient Management

All patients were intubated, and continuous hyperventilation was induced with $Paco_2$ between 25 and 35 mm Hg. All received fluid resuscitation at 1 to 2 mL/kg/h. If cerebral perfusion pressure (mean arterial blood pressure − ICP) was

less than 60 mm Hg, adequate amounts of albumin were given. To maintain urine output at above 0.5 mL/kg/h, adequate amounts of colloid fluids and continuous infusion of dopamine at 3 to 5 μg/kg/min were administered as needed during the study period. No corticosteroids or mannitol were administered during the study. After initial resuscitation, all patients were immediately subjected to computed tomographic study of the head. An intraventricular catheter was then placed to provide continuous ICP and intracranial temperature monitoring. If necessary, intracranial mass lesions associated with midline displacement greater than 5 mm were surgically evacuated. Two of nine patients in the mild hypothermia group and one of eight patients in the normothermia group required craniotomy. Ventriculostomy was performed in all 17 patients.

In all patients, intracranial hypertension was managed initially by conventional ICP reduction therapies such as cerebrospinal fluid drainage, mild hyperventilation, and administration of high-dose barbiturates. Barbiturate therapy was initiated by intravenous injection of thiopental at 4 to 6 mg/kg followed by continuous infusion at 6 to 8 mg/kg/h to maintain a burst-suppression electroencephalography pattern. If the ICP remained below 20 mm Hg after high-dose barbiturate therapy, the patient was assigned randomly to the HT or NT group.

After group assignment, mild hypothermia (intracranial temperature, 34°C) was induced in the HT group as quickly as possible by initiating surface cooling, which was accomplished by placing water-circulating blankets above and below the patient. Intracranial temperature, measured in the lateral ventricle, was maintained at 33.5° to 34.5°C. Mild hypothermia was maintained for 48 hours by continuous infusion of barbiturates at 6 to 8 mg/kg/h. When the therapy was discontinued, the patient was rewarmed slowly (approximately 1.0°C per day) by continuous infusion of barbiturates at 2 mg/kg/h to prevent shivering. On day 5, intracranial temperature reached 37°C in the HT group. When rewarming was complete, the barbiturates were gradually withdrawn.

Normothermia (intracranial temperature, 37°C) was initiated in the NT group immediately after randomization. Intracranial temperature was maintained at 36.5° to 37.5°C by surface cooling for 5 days. After these 5 days, we maintained all 17 patients at 37.5° to 38.5°C by surface cooling. To match conditions between the two groups, barbiturates were infused continuously at 6 to 8 mg/kg/h during the initial 48 hours, followed by continuous infusion of barbiturates at 2 mg/kg/h to prevent shivering. After completion of the protocol, the barbiturates were gradually withdrawn.

Blood Samples and Quantification of HSP Expression in PMNLs

The date of injury was defined as day 0. Blood samples were obtained for three time periods; days 0 to 1, days 2 to 5, and days 6 to 14 after injury. Expression of HSPs in PMNLs was evaluated for each sample.

PMNLs were isolated from heparinized peripheral blood by 6% dextran sedimentation (Sigma Chemical Co., St. Louis, MO), Ficoll-Hypaque density-gradient centrifugation (Sigma), and hypotonic lysis of the remaining red blood cells. PMNLs isolated according to this procedure were confirmed by trypan-blue exclusion to be more than 95% pure and more than 95% viable. PMNLs were washed twice in RPMI 1640 (Nakarai, Kyoto, Japan) by centrifugation, resuspended at 1×10^6 cells/mL in RPMI 1640 containing 10% fetal calf serum (Sigma) and 10% dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and stored at −80°C until assay.

Flow cytometric analysis of HSP expression in PMNLs was performed by means of an indirect immunofluorescence staining technique with monoclonal antibodies to HSP27, HSP60, HSP70, and HSP90 (Affinity Bioreagents, Inc., Golden, CO) according to the method of Chant et al. but with some modifications.[9] Briefly, cells were fixed with 1% paraformaldehyde in phosphate-buffered saline (Mo Bio Laboratories Inc., Nagoya, Japan) for 10 minutes at room temperature and washed twice with phosphate-buffered saline. PMNLs were resuspended at a concentration of 1×10^5 cells/100 μL and stained with anti-HSP27 (5 μL), anti-HSP60 (5 μL), anti-HSP70 (5 μL), or anti-HSP90 (5 μL) for 3 hours at room temperature in the presence of 0.25% saponin (Sigma), which permeabilizes the cell membrane. Cells were washed twice in phosphate-buffered saline containing 2% bovine serum albumin (Sigma) and incubated with fluorescein isothiocyanate–conjugated goat antimouse immunoglobulin G (Sigma) diluted to 1:200 for 30 minutes in the dark at room temperature. Cells were then washed twice with phosphate-buffered saline, and immunofluorescence was analyzed by flow cytometry (Coultier Epics Elite, Coultier Co., Miami, FL). Fluorescence data were obtained in a linear 1,024-channel mode according to the fluorescence intensity of greater than 3,000 individual cells. Results are presented as the mean fluorescence intensity per cell.

Complications and Patient Outcome

Pneumonia was diagnosed when a patient met at least three of the following criteria: new infiltrates on chest x-ray films, purulent tracheobronchial secretions, positive pathogenic bacterial culture from tracheobronchial secretions, and impaired pulmonary gas exchange. Meningitis was diagnosed when white blood cell (WBC) counts in the cerebrospinal fluid were greater than 100 cells/L.

The outcome of each patient was assessed 6 months after injury according to the Glasgow Outcome Scale (GOS) as follows: 1, death; 2, vegetative state; 3, severe disability; 4, moderate disability; and 5, mild or no disability. For statistical comparison, patients with a GOS score of 4 or 5 were classified as having a favorable outcome, and those with a GOS score of 1, 2, or 3 were classified as having an unfavorable outcome. Each survivor participated in a personal follow-up interview by clinic visit or by telephone.

In Vitro Study

Blood samples were obtained from 10 healthy volunteers. The method of PMNL isolation from peripheral blood was as described above. PMNLs were incubated at 37°C, 34°C, or 26°C (room temperature) for 1 hour with sodium arsenite, an inducer of HSP. We selected sodium arsenite as an inducer of HSP because it has been shown to induce more major HSPs in comparison with other inducers such as heavy metals, iodoacetamide, sulfhydryl oxidizing agents, ethanol, and other aliphatic alcohols.[10,11] We examined the effects of several concentrations of sodium arsenite on HSP expression in PMNLs. We selected a concentration of 100 μmol/L because the expression of four HSPs was most enhanced in viable PMNLs at this concentration.

PMNLs incubated for 1 hour under this procedure were confirmed by trypan-blue exclusion to be more than 95% viable. The cells were washed twice and stored in the same solution used in our clinical study at −80°C until assay. Flow cytometry was then used to measure the expression of HSP27, HSP60, HSP70, and HSP90.

Statistical Analysis

Results are shown as mean ± SD. Unpaired Student's *t* test was used to analyze differences between patients and healthy volunteers at the various time points, and analysis of variance was used to analyze differences between time points. Clinical characteristics and complications were compared between the two groups by Fisher's exact test. In the in vitro study, results between groups were evaluated statistically by one-way analysis of variance. Statistical significance was assigned at $p < 0.05$.

RESULTS

Patient Characteristics

Clinical characteristics of the patients in the two groups are summarized in Table 1. The two groups did not differ significantly in age, Glasgow Coma Scale score at admission, neurologic status, ICP level after conventional therapy, additional injuries, or Injury Severity Score. The incidence of hypotension at admission, defined as a sustained decrease in systolic blood pressure to 100 mm Hg, was similar in the two groups (observed in zero patients in the HT group and in one patient [12.5%] in the NT group). The incidence of hypoxia at admission, defined as a Pao_2 less than 60 mm Hg, was similar in the two groups (two patients [22.2%] in the HT group and two patients [25.0%] in the NT group).

HSP Expression Levels in PMNLs

Expression of HSPs in PMNLs from both groups and from healthy volunteers is shown in Figure 1. Expression of HSP60 in the HT group was significantly lower over all three time periods (days 0–1, days 2–5, and days 6–14) than that in the NT group ($p < 0.05$). Expression of HSP60 in the NT group was significantly greater over all three time periods than that in the healthy volunteers ($p < 0.05$), and that in the HT group was similar to that in the healthy volunteers (Fig. 1*B*). Expression of HSP70 in PMNLs from the HT group and the NT group was significantly greater over all three time periods than that in the healthy volunteers ($p < 0.05$) (Fig. 1*C*), but there were no significant differences between the HT and NT groups. There were no significant differences in the expression of HSP27 or HSP90 in PMNLs from the HT group, the NT group, and healthy volunteers (Fig. 1*A* and *D*).

Infectious Complications

As shown in Table 2, five of nine patients in the HT group suffered from pneumonia during the first week, whereas none of the patients in the NT group exhibited this complication ($p = 0.020$). There was no correlation between the incidence of pneumonia and the length of barbiturate coma in each group. Seven patients in the HT group and two patients in the NT group suffered from meningitis during the first week ($p = 0.045$). Only one of nine patients in the HT group showed no infectious complications during the first week, whereas six of eight patients in the NT group exhibited no complications ($p = 0.013$).

Clinical Outcome

Patient outcomes are shown in Table 1. In the HT group, the outcomes at 6 months after injury included good recovery in five patients, moderate disability in one, severe disability in two, and death in one. One patient died of pneumonia at a rehabilitation hospital at 3 months postinjury. In the NT group, the outcomes at 6 months after injury included good recovery in five patients, moderate disability in two, and severe disability in one. Six of nine patients (67%) in the HT group and seven of eight patients (87.5%) in the NT group achieved a favorable outcome.

In Vitro Study

The expression of HSP60 in PMNLs from healthy volunteers in the presence of sodium arsenite at 26°C and 34°C was significantly lower than that at 37°C ($p < 0.05$) (Fig. 2*B*). There were no differences in the expression of HSP27, HSP70, or HSP90 at 37°C, 34°C, or 26°C ($p < 0.05$) (Fig. 2*A*, *C*, and *D*).

DISCUSSION

We previously showed that mild hypothermia should not be used for the treatment of severely head-injured patients with low ICP.[4] However, we do not believe that mild hypothermia is entirely useless in treating severely head-injured patients. We stress aggressive use of mild hypothermia to treat severely head-injured patients with high ICP, because the most consistent finding with the use of this method has been a reduction in ICP.[12–15] Although Clifton et al. concluded in a National Institutes of Health multicenter study that treatment with hypother-

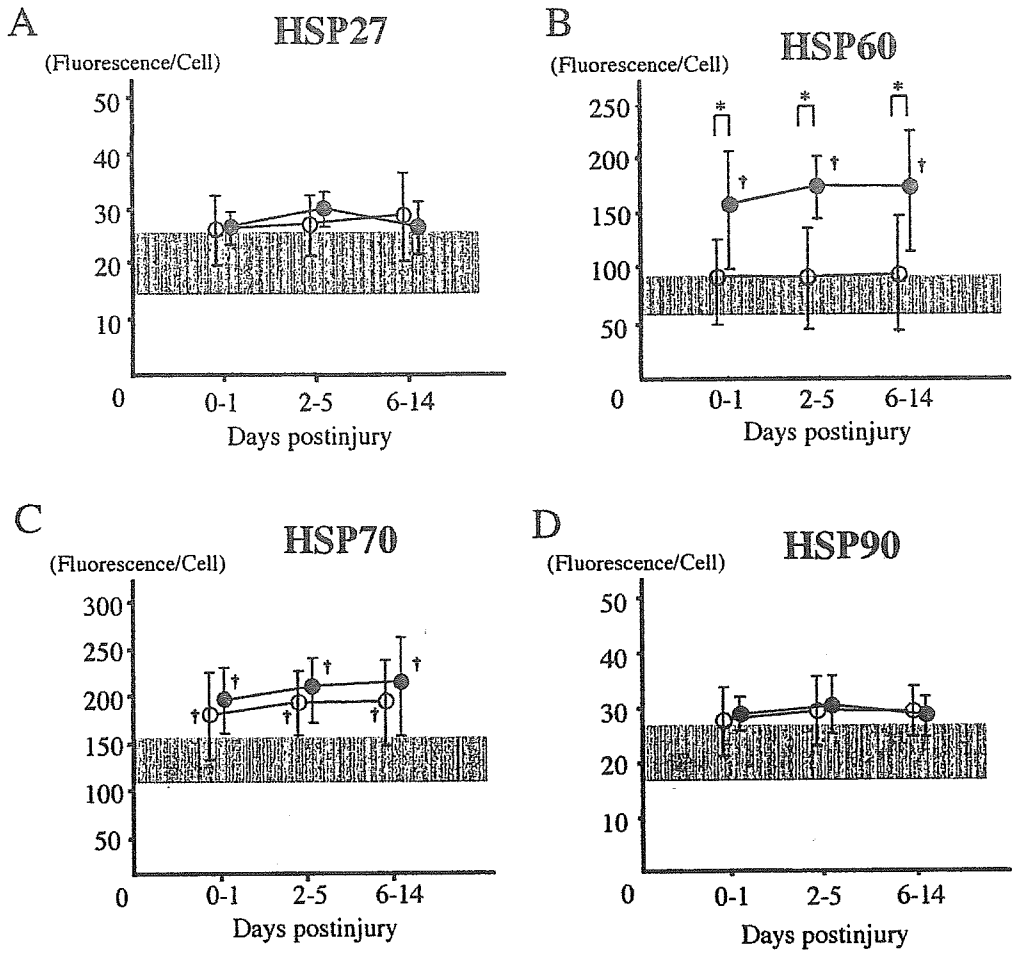

Fig. 1. *Changes in the expression of HSP27* (A), *HSP60* (B), *HSP70* (C), *and HSP90* (D) *in PMNLs from severely head-injured patients. (○) Mild hypothermia group; (●) normothermia group. Values are expressed as mean ± SD;* $^{*}p < 0.05$ *compared with the NT group;* $\dagger p < 0.05$ *compared with healthy volunteers. The* gray area *indicates the mean ± SD of healthy controls.*

mia at a body temperature of 33°C within 8 hours after injury is not effective in improving the outcome of patients with severe brain injury, they did report a beneficial effect of mild hypothermia on high ICP.[16] Therefore, we believe that mild hypothermia should be used to decrease ICP in patients with high ICP refractory to conventional therapies.

Table 2 Infectious Complications in 17 Patients within 1 Week after Severe Head Injury

HT (34°C) Group			NT (37°C) Group		
Patient	Pneumonia	Meningitis	Patient	Pneumonia	Meningitis
1	+	+	1	–	–
2	+	+	2	–	–
3	+	+	3	–	+
4	–	+	4	–	–
5	–	–	5	–	+
6	–	+	6	–	–
7	+	–	7	–	–
8	–	+	8	–	–
9	+	–			

Patients with Infections	HT Group	NT Group	*p* Value
Total	8	2	0.013
With pneumonia	5	0	0.020
With meningitis	7	2	0.045

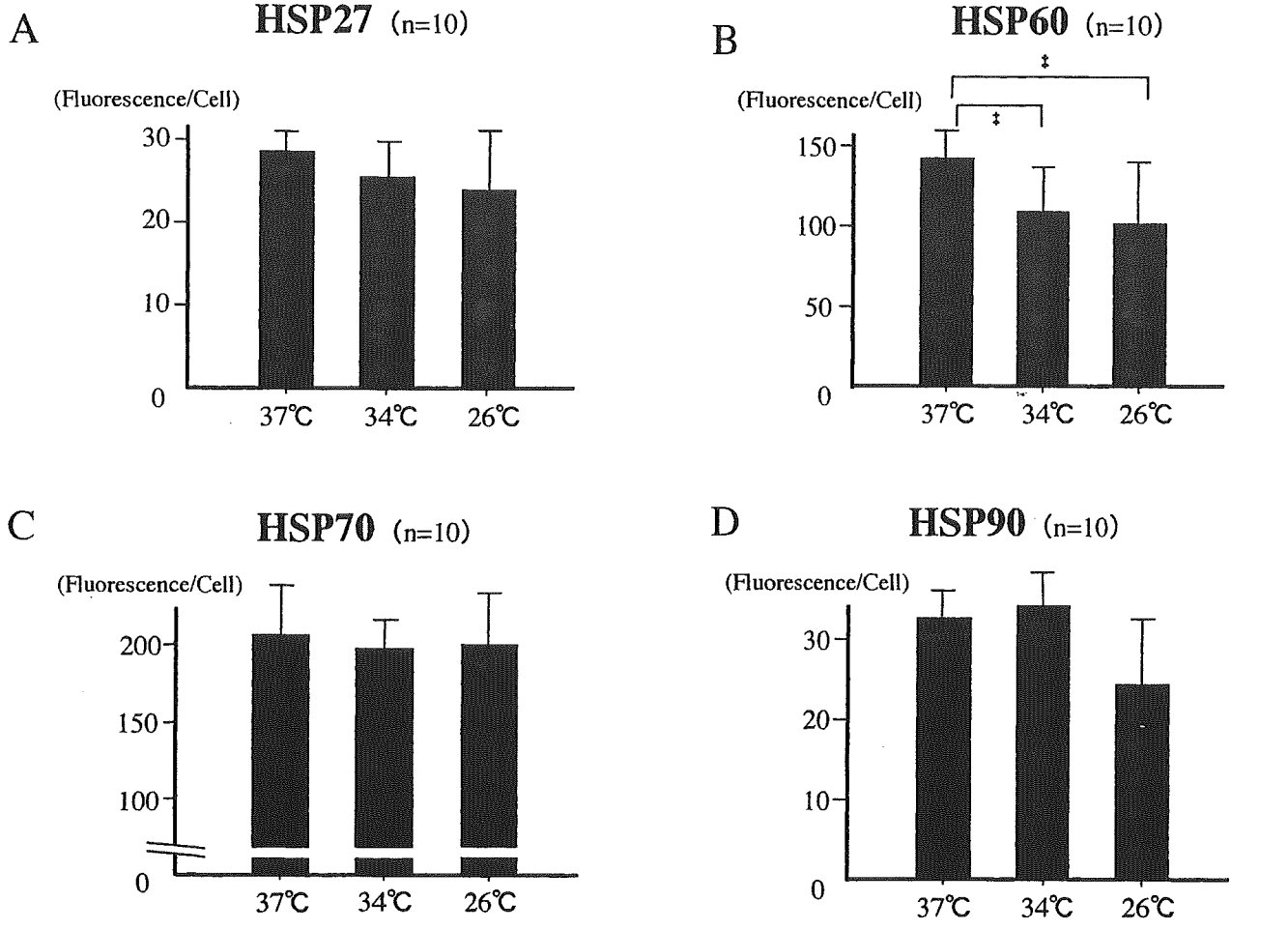

Fig. 2. *The expression of HSP27 (A), HSP60 (B), HSP70 (C), and HSP90 (D) in PMNLs from healthy volunteers at 37°C, 34°C, and 26°C in our in vitro study. Values are expressed as mean ± SD; ‡p < 0.05 compared with that of 37°C.*

Although mild hypothermia is useful in decreasing high ICP in severely head-injured patients, mild hypothermia itself markedly increases the risk of infection.[4] Many investigators have suggested that this susceptibility to infection may reflect on effect on WBC function. For example, Biggar et al. showed that in vitro neutrophil chemotaxis under agarose was significantly impaired at 29°C and that neutrophil and monocyte migration in vivo was also markedly reduced at 29°C.[17] Akriotis and Biggar reported that when human and porcine neutrophils are exposed to hypothermia (29°C) in vitro, neutrophil functions, such as fighting bacteria, phagocytosis, oxidative metabolism, and migration, were compromised at 29°C versus 37°C.[18] However, PMNL function, such as phagocytosis and oxidative activity, did not differ between the HT (34°C) and the NT (37°C) groups (unpublished data). Therefore, we focused on HSP60, which is believed to play an essential role in innate immunity.

HSPs are highly conserved among both prokaryotes and eukaryotes. HSPs are grouped into families on the basis of molecular weight and amino acid sequence homology. Although HSPs are up-regulated under a number of stress conditions, many are expressed constitutively in normal cells. HSPs play essential roles in the folding, unfolding, and transport of proteins. Heterologous HSPs are antigens in many infectious diseases involving bacteria, parasites, and fungi. In particular, HSP60, a well-conserved and immunodominant antigen that elicits a cellular and humoral immune response, may play a role in host defense against invading microorganisms and in autoimmune disorders.[19]

The ability of either chlamydial or human HSP60 to activate human vascular cells and to trigger NF-κB activation suggests a novel amplification loop in arterial inflammation.[20] HSP60 has the following unique features: ubiquitous distribution in the biosphere; extraordinary homology between bacterial and mammalian forms; and its abundant expression under stress situations that occur during infection.[21] HSP60 has been found to be a common antigen of many bacterial pathogens including *Borrelia*, *Legionella*, *Chlamydia*, *Coxiella*, *Salmonella*, *Treponema*, *Rickettsia*, *Mycobacterium*, and *Pseudomonas* species.[22] A number of observations suggest that T cells specific for HSP60 are involved in the phenomena associated with organ-specific inflammatory disease, suggesting that many of the T cells that recognize bacterial HSP60 also react with endogenous HSP60.[23,24] Bacterial HSP60 is termed a "common antigen"

because, in individuals with bacterial infections, antibodies against bacterial HSP60 have been found, and antisera from infected individuals cross-react with HSP60 from other bacteria. It appears, therefore, that the body handles infectious diseases by increasing the expression of HSP60. In the present study, the expression of PMNL HSP60 in the HT group was significantly reduced during the initial 2 weeks after head injury, which appears to relate to the finding that the incidence of infectious complications was significantly higher in the HT group than in the NT group ($p < 0.05$).

To exclude influence on the immune system caused by the severity of the head injury itself or by various medications such as barbiturates, we performed an in vitro study using blood samples obtained from 10 healthy volunteers. The expression of HSP60 in PMNLs incubated at 34°C or at 26°C was significantly lower than that in cells incubated at 37°C. No differences were observed in the expression of HSP27, HSP70, or HSP90 at 37°C, 34°C, or 26°C ($p < 0.05$). These results show clearly that reduced expression of PMNL HSP60 in the HT group was attributable to the decrease in body temperature.

We are currently studying WBC function, specifically, the expression of HSPs in lymphocytes and monocytes, and activation of NF-κB in PMNLs. Further study is needed to address the cause of increased infectious complications in patients receiving mild hypothermia.

In conclusion, we have shown that expression of HSP60 in PMNLs from the HT group was significantly lower than in those from the NT group during the initial 2 weeks ($p < 0.05$), whereas no significant differences in expression of HSP27, HSP70, or HSP90 were identified between the two groups. The incidence of infectious complications was significantly higher in the HT group than in the NT group ($p < 0.05$). In the in vitro study, only the expression of HSP60 at 26°C and 34°C was significantly lower than at 37°C, whereas the expression of the other HSPs did not differ significantly at 26°C, 34°C, or 37°C. We conclude that mild hypothermia reduces the expression of HSP60 in PMNLs in severely head-injured patients, and that this reduced expression may suppress innate immunity.

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Profile of Gene Expression in the Subventricular Zone after Traumatic Brain Injury

KAZUHISA YOSHIYA, HIROSHI TANAKA, KOUSUKE KASAI, TARO IRISAWA, TADAHIKO SHIOZAKI, and HISASHI SUGIMOTO

ABSTRACT

Neural stem cells, which reside in the subventricular zone (SVZ) and dentate gyrus (DG) of adult mammals, give rise to new neurons throughout life. However, these neural stem cells do not appear to contribute to regeneration in the damaged central nervous system. Following traumatic brain injury (TBI) in adult rats, the number of proliferating cells labeled with bromodeoxyuridine (BrdU) is significantly increased in the bilateral SVZ and DG; however, these proliferating cells do not contribute to effective regeneration in the damaged area. To gain insight into the molecular mechanisms of these biological actions, changes in gene expression in the SVZ after brain trauma were examined by cDNA microarray. Of 9,596 genes screened, 97 were upregulated and 204 were downregulated. Classifying these genes according to their function suggests that TBI affects a broad range of cellular functions. The validity of the data was confirmed by RT-PCR. The expression of some genes localized in the SVZ was confirmed by *in situ* hybridization. This combined strategy is effective for comprehensive analysis of the pathophysiological changes in the SVZ after brain injury and should contribute to the understanding of the molecular events that occur after injury. In the future, this may enable regeneration of the damaged central nervous system.

Key words: cDNA microarrays; *in situ* hybridization; neural stem cell; RT-PCR; subventricular zone; traumatic brain injury

INTRODUCTION

NEURAL STEM CELLS (NSCs), which have self-renewal and multilineage potentials, have been identified in the embryonic and adult mammalian brain (Weiss et al., 1996; McKay, 1997). In the adult mammalian brain, NSCs have been identified in limited areas, such as the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles (Altman, 1969; Altman and Das, 1965; Lois and Alvarez-Buylla, 1993). Precursor cells in these two areas can give rise to new neurons throughout life. New neurons from the SGZ migrate into the DG granule cell layer, and those from the SVZ migrate via the rostral migratory stream into the olfactory bulb (Lois et al., 1996) and also enter into the association neocortex (Gould et al., 1999).

Recent studies have shown that neurogenesis and synaptic plasticity of these cells are influenced by stress (Gould and Tanapat, 1999), environmental enrichment (Kempermann et al., 1997), and physical exercise (van Praag et al., 1999). Neurogenesis also occurs in the SGZ and SVZ after insult to the central nervous system (CNS),

Department of Traumatology and Acute Critical Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, Japan.

such as traumatic brain injury (TBI) (Dash et al., 2001; Gould and Tanapat, 1997; Kernie et al., 2001; Yoshimura et al., 2001), ischemic brain injury (Jin et al., 2001; Liu et al., 1998), seizures (Parent et al., 1997), and other stresses (Magavi et al., 2000). Although brain lesions appear to facilitate neurogenesis from NSCs in the adult CNS, suggesting that the damaged brain can self-repair, NSCs alone are not sufficient for regeneration of the CNS. If we hope to manipulate endogenous adult neural precursor cells to give rise to new neurons for regeneration, we must understand the genes and molecules that regulate cell division and the fate of neural precursor cells (Cattaneo and McKay, 1991).

The present study addresses how TBI affects cells in the SVZ and DG, where NSCs are located. Interestingly, proliferation of these cells was stimulated after injury, whereas regenerating neuronal cells were not observed around the injury site. To gain insight into the molecular mechanisms regulating these biological actions in the SVZ after injury, a combined strategy of cDNA microarray screening followed by RT-PCR and *in situ* hybridization was used. Such comprehensive analyses will help pinpoint the genes important in the pathogenesis of brain injury.

MATERIALS AND METHODS

Controlled Cortical Impact Injury

All protocols involving the use of animals were approved by the Institute of Experimental Animal Sciences (IEXAS) and were in compliance with the Guidelines for the Care and Use of Laboratory Animals of the Osaka University Medical School Animal Care and Use Committee. A controlled cortical impact device was used to cause TBI as described previously (Dixon et al., 1991). Briefly, adult male Wistar rats weighing 300–350 g were anesthetized with sevoflurane and chloral hydrate (400 mg/kg, i.p.) and placed in the stereotaxic frame of the injury device in a prone position. A middle incision was made, the soft tissues were reflected, and a 10-mm craniectomy was made centrally between the bregma and lambda, with the medial edge of the craniectomy 1 mm lateral to the midline. Moderate cortical injury was induced with a 5-mm impactor tip with 2-mm deformation at a speed of 6 m/sec at an angle of 30° from the vertical plane. Sham-operated rats were similarly anesthetized, and craniectomy was performed without cortical injury. After the injury or sham operation, the skull was replaced, the scalp was sutured, and the rats were allowed to recover.

BrdU Administration

BrdU (Sigma, Steinheim, Germany) was dissolved in saline at a concentration of 10 mg/mL and injected i.p. 24 h before animals were killed (150 mg/kg; three times as usual). This allowed the measurement of the number of mitotic cells during a 24-h period and provided an index of the rate of cell birth at a specific time point after TBI.

Tissue Preparation and Immunohistochemistry

Animals were anesthetized as described above. They were perfused transcardially with saline, then by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, post-fixed in 4% PFA overnight, and placed in 20% sucrose overnight. Coronal sections (25 μm) were placed on silane-coated slides. For immunohistochemical detection of BrdU-labeled nuclei, DNA was denatured to expose the antigen. Before incubation with anti-BrdU primary antibody, sections were pretreated in 50% formamide/2 × SSC (sodium chloride/sodium citrate) at 65°C for 2 h, incubated at 37°C for 30 min in 2 N HCl, and rinsed for 10 min at 25°C in 0.1 M boric acid, pH 8.5. Sections were then incubated in 0.3% H_2O_2 to block endogenous peroxidase activity and finally incubated for 2 h at room temperature in PB with 5% goat serum, 0.1% bovine serum albumin, and 0.3% Triton X-100 (blocking buffer). Sections were incubated overnight at 4°C in mouse monoclonal anti-BrdU antibody (Sigma) diluted 1:500 in blocking buffer. Sections were washed in PB three times and then incubated with biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 2 h at room temperature. After three 5-min rinses in PB, sections were placed in avidin-peroxidase complex solution (Vector Laboratories) for 2 h. After two additional 10-min washes in PB and one additional 10-min wash in 0.05 M Tris-buffered saline (TBS), sections were incubated for 2 min in peroxidase reaction solution (0.2 mg/mL diaminobenzidine, 0.01% H_2O_2, 1.2% $NiCl_2$, and 0.05 M TBS). Processing was stopped with 0.05 M TBS, and sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped in enthelan. Sections were examined with an Olympus BX50 microscope (Olympus, Tokyo, Japan).

Cell Counting

BrdU-positive cells were counted blindly in five DAB-stained 25-μm coronal sections, spaced 200 μm apart per animal. Cells were counted under high power on an Olympus BX50 microscope fitted with a magnifier digital camera HC-2500 (Fujifilm, Tokyo, Japan), and each image was displayed on a computer monitor. Results

were expressed as the average number of BrdU-positive cells per section and reported as the mean ± SEM.

RNA Isolation

On day 4 after brain injury, rats were killed with excess anesthesia, and the periventricular tissues from the injured or sham-operated site were dissected. These tissues were homogenized in Isogen RNA extraction reagent (Nippon Gene, Tokyo, Japan) with a Biotron homogenizer (Biotron, Sydney, Australia) for 15 sec prior to RNA isolation. Total RNA was prepared from homogenized tissue with Isogen reagent according to the manufacturer's instructions. mRNA was isolated from total RNA with oligo dT columns and standard Oligomix (Takara, Shiga, Japan). The quality of extracted total RNA and mRNA was confirmed with an Agilent Technologies 2100 Bioanalyzer-Bio Sizing (Agilent Technologies, Palo Alto, CA). Each pool of mRNA used for microarray analysis was derived from eight rats.

Microarray Hybridization and Analysis

A LifeArray chip of the LifeArray System (Incyte Genomics, Palo Alto, CA), which contains 9596 cDNAs, was used for microarray analysis. cDNA from injured rats was cyanine 3 (Cy3)–labeled by reverse transcription from 200 ng mRNA by means of a LifeArray Probe Labeling Kit (Incyte Genomics) according to the manufacturer's instructions, and cDNA from sham-operated (control) rats was cyanine 5 (Cy5)–labeled. Two different dye-labeled cDNA probes were hybridized simultaneously with one cDNA chip at 60°C for 6 h with a LifeArray hybridization chamber, hardware kit, and wash solutions (Incyte Genomics) according to the manufacturer's instructions. After hybridization, scanning of the two fluorescent intensities of the cDNA chip was performed with a standard two-color microarray scanner (GenePix 4000A DNA Microarray Scanner, Axon Instruments, Union City, CA). Differential gene expression was profiled with GemTools software (Incyte Genomics).

TABLE 1. SEQUENCES OF PRIMER PAIRS USED FOR RT-PCR

Gene name	*Sequences (5′ → 3′)*	*Product size*
Calm2		
Forward	GCATGGCTGACCAACTGACTGAAGA	547 bp
Reverse	TAGGGGGGGGGAACCTTTTACAGAT	
Gelsolin		
Forward	ATGTGCAGGTGGAAGAAGGCAGTGA	518 bp
Reverse	AGATGGACAACCCTCAAAGGCACTG	
MLP		
Forward	CATCATGGGCAGCCAGAGCTCCAAG	636 bp
Reverse	AGCTTAGAGATCACCCACCAGCCCC	
Cnil		
Forward	CATGGGTTCACCTTCGCAGCATTC	683 bp
Reverse	GAGCTTTGCCTGGCCCTAGTCAGAA	
MT-3		
Forward	AACTAAGCTACAGTCTCTCGCGGCT	336 bp
Reverse	TTATTCACACGAGGCGGAGCAGGAA	
Bak		
Forward	TCATTCAGGTGACAAGTGACGGTGG	734 bp
Reverse	TAGGGAGAGAGGTTTAGTCCAGCCA	
PSD-95		
Forward	ACGATGATCTTCTCTCCGAGTTCCC	600 bp
Reverse	TATTCAGTCCACACCAAGGGCCCAT	
Neurochondrin		
Forward	AATGTCGTGTTGTGACCTGGCTGCG	469 bp
Reverse	GTAGGTGTCATCGATCATGGAGCGG	
NELF		
Forward	AACCCTTTCAGCTTCCAGACGGCAA	770 bp
Reverse	AGGTTCTGGAATAGCTTCTCCCCGT	

Reverse Transcription–PCR

Total RNA (5 μg) was reverse transcribed with oligo (dT) by reverse transcriptase from Moloney murine leukemia virus (Invitrogen Corp., Carlsbad, CA). For PCR amplification, specific oligonucleotide primers (10 pmol each) were incubated with 1 μL of cDNA template (1 μg/μL) in a 50-μL PCR reaction mixture containing 1.5 mM $MgCl_2$, 25 mM KCl, 10 mM Tris, pH 9.2, mixed deoxynucleotides (1 mM each), and 1 unit of Taq polymerase; the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) was used. PCR amplification conditions were 1 cycle at 94°C for 2 min; 30 cycles at 94°C for 30 sec, 50–63°C for 30 sec, and 72°C for 60 sec; and 1 cycle at 72°C for 5 min. Reaction products were resolved by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining and UV transillumination. PCR primers were prepared according to the Entrez Nucleotides database as described in Table 1 (www.ncbi.nlm.nih.gov/entrez/query.fcgi). The product of constitutively expressed β-actin mRNA served as the internal standard. Representative genes are calmodulin 2 (Calm2), gelsolin, myristrated alanine-rich C kinase substrate (MARCKS)–like protein (MLP), cornichon-like protein (Cnil), metallothionein 3 (MT-3), bcl2 homologous antagonist/killer protein (Bak), discs, large homolog 4 (PSD-95), neurochondrin, and nasal embryonic LH-RH factor (NELF).

In Situ Hybridization

Brains were removed from rats 4 days after the injury or sham operation, rapidly frozen on dry ice, cryostat sectioned (14 μm), thaw-mounted on slides, and stored at −80°C. *In situ* hybridization was performed for four genes with more than twofold changes in expression on microarrays. Antisense and sense RNA probes were transcribed *in vitro* after PCR. PCR was performed according to the same procedure and primers described above. PCR products of Calm2, gelsolin, MLP, and MT-3 were subcloned into the pGEM-T vector (Promega, Tokyo, Japan) and maintained in DH5α *E. coli*. Each plasmid was purified and sequenced by standard procedures. To synthesize hybridization riboprobes by *in vitro* transcription, pGEM-T vector containing Calm2 cDNA was linearized with NdeI (antisense probe) or NcoI (sense probe), and pGEM-T vectors containing gelsolin, MLP, and MT-3 cDNA were linearized with NcoI (antisense probe) or NdeI (sense probe). The linearized cDNA was then incubated at 37°C for 60 min with a mixture consisting of 2 μL 5 × transcription buffer, 0.5 μL 100 mM dithiothreitol, 0.5 μL RNase inhibitor, 1 μL 10 × DIG labeling mix (Roche, Basel, Switzerland), 0.5 μL cDNA template (1 μg/μL), and 1 μL appropriate RNA polymerase (T7 or SP6). DNA was digested with 1 μL DNase and incubated at 37°C for 15 min. Integrity of the synthesized probes was confirmed by electrophoresis, and the concentration was determined with a spectrophotometer. Sections were fixed in 4% PFA in 0.1 M PB for 20 min. After two washings with 0.1 M PB, sections were treated with 10 μg/mL of proteinase K in 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA (pH 8.0) for 2 min at room temperature. Sections were again fixed, acetylated with acetic anhydrate in 0.1 M triethanolamine, rinsed

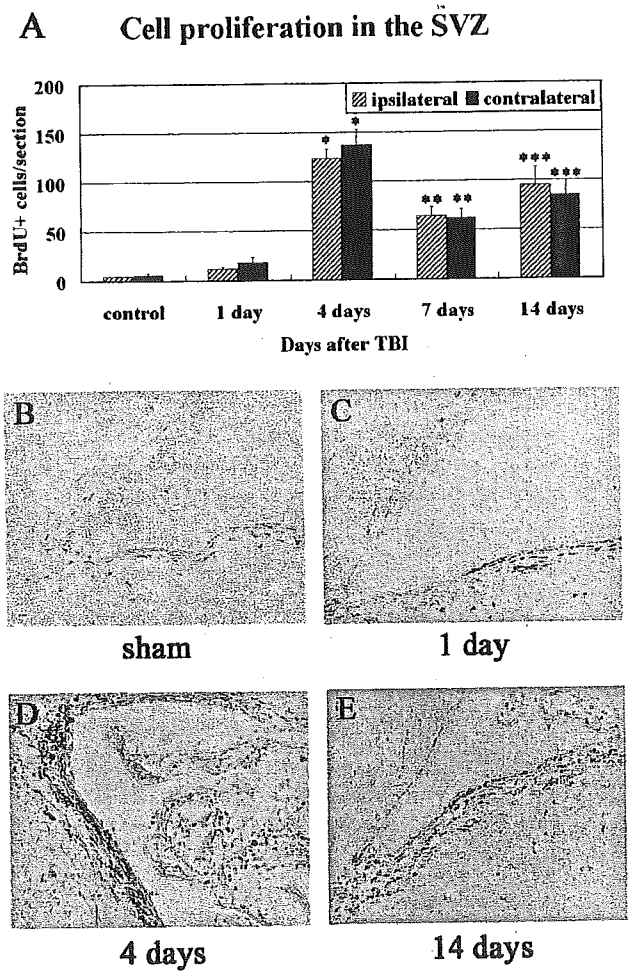

FIG. 1. Cell proliferation in the subventricular zone (SVZ) after traumatic brain injury (TBI). (**A**) Cell proliferation in the SVZ was significantly increased at 4 days after TBI and persisted until 14 days. Asterisks indicate significant differences in comparison to control values; $*p < 0.001$; $**p < 0.01$; $***p < 0.05$. (**B**) Baseline mitotic activity in the ipsilateral SVZ of sham-operated (control) rats. Numbers of BrdU-positive cells were not significantly increased at 1 day after TBI (**C;** ipsilateral). At 4 days after TBI, cell proliferation was increased (**D;** ipsilateral) and persisted until 14 days after TBI (**E;** ipsilateral). Bar in **B** = 100 μm.

with PB, dehydrated, and air dried. DIG-labeled RNA probes (antisense or sense) were diluted in hybridization buffer, placed over the sections, and covered with silane-coated coverslips. Hybridization was performed for 36 h in a humidified chamber at 50°C. Hybridization buffer consisted of 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8.0), 10% dextran sulfate, 1 × Denhardt's solution, 0.2% sarcosyl, 500 μg/mL yeast tRNA, and 200 μg/mL herring sperm DNA. The probe concentration was 500 ng/μL per slide. After hybridization, slides were immersed in 4 × SSC at 57°C, and coverslips were allowed to fall off. Sections were then incubated at 57°C in 50% deionized formamide with 2 × SSC for 30 min. After three rinses with RNase buffer (0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 8.0) for 10 min each at 37°C, the sections were treated with 1 μg/mL RNase in RNase buffer at 37°C for 30 min. After an additional wash in RNase buffer, the slides were incubated in 50% formamide with 2 × SSC for 30 min at 57°C. The sections were rinsed in DIG buffer 1 (0.1 M Tris-HCl and 0.15 M NaCl) for 10 min at room temperature and then incubated in 1.0% blocking buffer (1.0% blocking reagent [Roche], 0.1 M Tris-HCl, 0.15 M NaCl) for 60 min at room temperature. After a brief washing with DIG buffer 1, slides were transferred to a humidified chamber and incubated with a 1:1000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (Roche) containing 1.0% blocking buffer and 0.2% Tween-20 overnight at room temperature. This was followed by four 15-min washes in DIG buffer 1 containing 0.2% Tween-20 and a 10-min wash in DIG buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). Visualization was performed with a color developing solution (nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in DIG buffer 3). The visualization was stopped after 16–24 h by a wash in DIG buffer 3 and 1 × Tris-EDTA buffer (TE). The sections were coverslipped with TE:glycerol (1:1) solution.

Statistical Analysis

Values are expressed as the mean ± 1 standard error of the mean (SEM). Differences between groups were examined by one-way analysis of variance (ANOVA) and Fisher's multiple comparison tests; p values < 0.05 were considered statistically significant.

RESULTS

TBI Promotes Cell Proliferation in the SVZ

In the SVZ, the site of cortical impact injury showed no significant histological signs of cell death, although extensive loss of neuronal cells was evident around the lesion (data not shown). We were interested in the effects of TBI on NSCs in the brain because NSCs are a potential therapeutic target for treatment of brain injury. To examine whether cortical injury influences the status of NSCs in brain, we quantified cell proliferation in the SVZ and DG, which are the regions where neural precursor cells reside. We quantified changes in BrdU labeling in these areas after TBI. In the SVZ, the number of BrdU-labeled nuclei increased and peaked

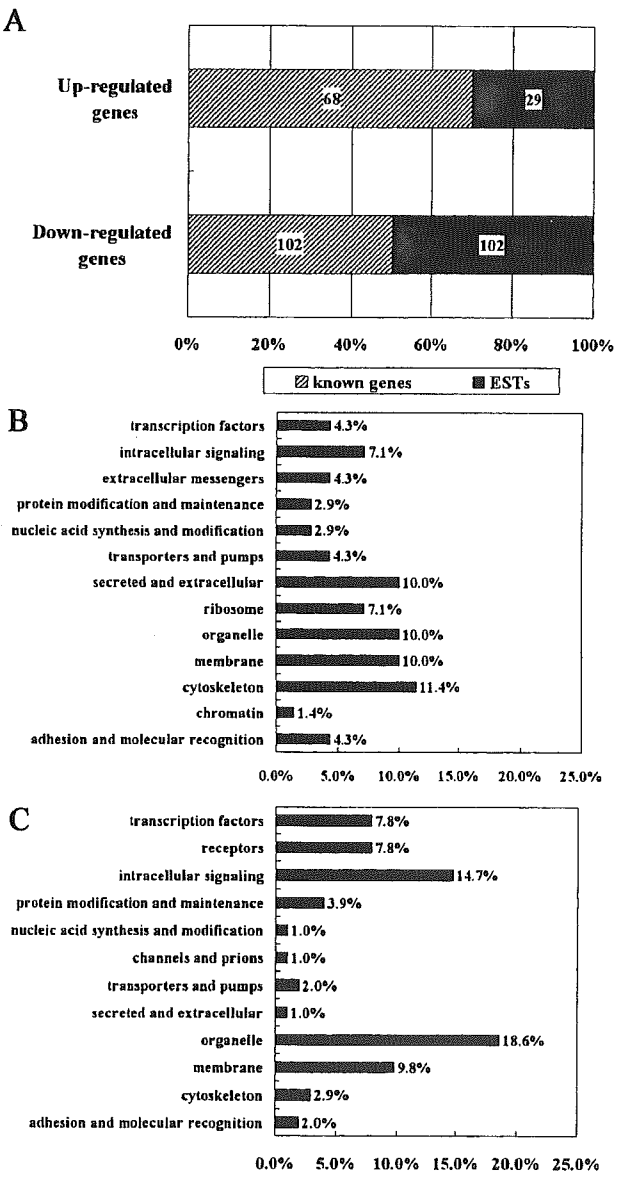

FIG. 2. (**A**) Numbers of upregulated and downregulated genes. (**B**) Categorization of upregulated genes according to function. (**C**) Categorization of downregulated genes according to function.

TABLE 2. FUNCTIONAL CLASSIFICATION OF KNOWN GENES UP-REGULATED AFTER TBI

Gene name	*Accession number*	*Fold change*
Transcription factors		
Jun proto-oncogene related gene d1	AA098137	2.3
Runt related transcription factor 3	AI608086	2.2
Undifferentiated embryonic cell transcription factor 1	AA547242	2.1
Intracellular signaling		
Calmodulin 2	AA575501	2.6
MARCKS-like protein	W54626	2.4
Cyclic nucleotide phosphodiesterase 1	AA276865	2.1
Aplysia ras-related homolog G (RhoG)	AI390848	2.0
Mitogen activated protein kinase 3	W36966	2.0
Extracellular messengers		
Angiotensinogen	AA571053	2.6
Endothelin 1	AA792278	2.2
Glucose phosphate isomerase 1 complex	AA050875	2.0
Protein modification and maintenance		
Cathepsin D	W16244	2.6
Cystatin C	AA770768	2.0
Nucleic acid synthesis and modification		
Exonuclease 1	AA672342	2.2
0-6-methylguanine-DNA methyltransferase	AA097896	2.0
Transporters and pumps		
ATPase, H+ transporting, lysosomal (vacuolar proton pump) 16kD	AI325715	2.2
Glycine transporter 1	AA771321	2.1
Vesicular inhibitory amino acid transporter	AI893217	2.0
Secreted and extracellular		
Apolipoprotein E	AI325603	2.9
Angiotensinogen	AA571053	2.6
Phospholipid transfer protein	AA666595	2.4
Endothelin 1	AA792278	2.2
Complement component 1, q subcomponent, alpha polypeptide	AA145122	2.0
Cystatin C	AA770768	2.0
Glucose phosphate isomerase 1 complex	AA050875	2.0
Ribosome		
Ribosomal protein L8	AA065602	2.5
Ribosomal protein L13	AI325708	2.2
Ribosomal protein L36	AA674987	2.1
Ribosomal protein L18	AA051462	2.0
Ribosomal protein, large, P1	AA880995	2.0
Organelle		
Cathepsin D	W16244	2.6
Jun proto-oncogene related gene d1	AA098137	2.3
ATPase, H+ transporting, lysosomal (vacuolar proton pump) 16kD	AI325715	2.2
Histone gene complex 2	AA536636	2.2
Runt related transcription factor 3	AI608086	2.2
Undifferentiated embryonic cell transcription factor 1	AA547242	2.1
0-6-methylguanine-DNA methyltransferase	AA097896	2.0
Aldehyde dehydrogenase 2, mitochondrial	AA794082	2.0
Membrane		
Histocompatibility 2, Q region locus 7	AA122791	2.5
ATPase, H+ transporting, lysosomal (vacuolar proton pump) 16kD	AI325715	2.2
Glycine transporter 1	AA771321	2.1
Histocompatibility 2, T region locus 10	AA067069	2.1
Myelin basic protein	AI605718	2.1
Claudin 4	AI048040	2.0
Vesicular inhibitory amino acid transporter	AI893217	2.0

TABLE 2. FUNCTIONAL CLASSIFICATION OF KNOWN GENES UP-REGULATED AFTER TBI (CONT'D)

Gene name	*Accession number*	*Fold change*
Cytoskeleton		
Melanoma X-actin	AI594289	2.9
Gelsolin	AA980357	2.4
Actin, alpha 1, skeletal muscle	AA770902	2.2
Tubulin, beta 5	AI386014	2.2
Actin, alpha 2, smooth muscle, aorta	AA624460	2.1
Beta-spectrin 3	AA049581	2.0
Crystallin, mu	AA733693	2.0
Tubulin alpha 6	AA617612	2.0
Chromatin		
Histone gene complex 2	AA536636	2.2
Adhesion and molecular recognition		
Histocompatibility 2, Q region locus 7	AA122791	2.5
Myelin-associated glycoprotein	AI893951	2.5
Histocompatibility 2, T region locus 10	AA067069	2.1
Others		
Creatine kinase, brain	AA517889	2.9
Eukaryotic translation elongation factor	AI325603	2.8
Karyopherin (importin) alpha 4	AI020155	2.7
Gdi-1 mRNA for RhoGDI-1 complete cds	AA105950	2.3
Prosaposin	AA606949	2.3
Amyloid beta (A4) precursor-like protein 1	W83478	2.2
Cornichon (drosophila)-like	AA016502	2.2
Mettalothionein 3	AA049749	2.2
Protective protein for beta-galactosidase	AA607008	2.2
Alsolase 1, A isoform	AI892240	2.1
ATPase, class II, type 9A	AA510705	2.1
Bcl2 homologous antagonist/killer	W77320	2.1
Fatty acid synthase	AA760287	2.1
Ferritin heavy chain	AI892243	2.1
Macrophage migration inhibitory factor	W14053	2.1
Solute carrier family 1, member 1	W16288	2.1
AE-binding protein 1	W13866	2.0
Alpha-2HS-glycoprotein	AI386037	2.0
Aplysia ras-related homolog G (RhoG)	AI390848	2.0
D-dopachrome tautomerase	AA638944	2.0
Discs, large homolog 4 (Drosophila)	AA200993	2.0
Eukaryotic translation initiation factor 2 alpha kinase 1	AI595623	2.0
Leukemia/lymphoma related factor	AA014909	2.0
Nasal embryonic LHRH factor	AA388971	2.0
Neurochondrin	AA018015	2.0
Telomeric repeat binding factor 2	AA098594	2.0

Accession numbers listed are GenBank accession numbers.

at 4 days after TBI, and this increase was observed bilaterally (Fig. 1). In the DG, cell proliferation was observed as previously described (Kernie et al., 2001) (data not shown). These results indicate that local injury promotes proliferation of cells, presumably including NSCs, in remote areas.

TBI Increases and Decreases Gene Expression in the SVZ

Our observation suggests that BrdU-positive (proliferating) cells in the SVZ did not migrate or differentiate into neurons around the lesion and olfactory bulb within

14 days after TBI, though we could observe cells migrating along the rostral migratory stream in control rats (data not shown). Therefore, there may be mechanisms regulating cell proliferation and inhibition of neuronal commitment and cell migration in the SVZ after TBI. However, the molecular mechanisms underlying this phenomenon are unknown.

To examine possible genetic changes in the SVZ after TBI, we used a cDNA microarray technique to screen for altered gene expression. We performed a comprehensive survey of gene expression in rats 4 days after TBI. Gene expression was considered upregulated or downregulated if the change was greater than twofold. Screening of 9,596 genes identified 97 upregulated genes and 204 downregulated genes in the SVZ of injured rats (Fig. 2A). The upregulated genes included 68 (70%) known genes and 29 (30%) expressed sequence tags (ESTs). Downregulated genes comprised 102 (50%) known genes and 102 (50%) ESTs.

Functional Classification of Differentially Expressed Transcripts

To analyze the gene expression profile efficiently, we classified the known genes into 15 functional categories (Fig. 2B,C). Some genes were contained in more than one functional group. For example, cathepsin D was classified into both the protein modification and maintenance and organelle groups. Therefore, the sum of the percentages of genes in each group is more than 100%. Categorization of the upregulated genes after injury is shown in Figure 2B and suggests that brain injury influenced a broad range of cellular functions in the SVZ by inducing expression of various genes. Upregulated known genes are listed in Table 2, and upregulated ESTs are listed in Table 3. Downregulated known genes are listed in Table 4, and downregulated ESTs are listed in Table 5.

Confirmation by RT-PCR

Kubo et al. (2002) previously showed the validity of microarray data by confirming such data with RT-PCR. We performed RT-PCR assays of nine upregulated genes to confirm our data. Each assay was repeated three times. A comparison of the RT-PCR data for Calm 2, gelsolin, MLP, Cnil, MT-3, Bak, PSD-95, neurochondrin, and NELF in periventricular tissue of the injured site 4 days after TBI with RT-PCR data from periventricular tissue of sham-operated rats is shown in Figure 3. These data are consistent with our microarray data.

In Situ Hybridization

We performed *in situ* hybridization to localize the upregulated genes. Expression patterns of Calm2, gelsolin, MLP, and MT-3 mRNAs were compared between rats with TBI (4 days after injury) and controls (Fig. 4). A positive signal was not observed in any section hybridized with sense probes (data not shown). After TBI, expression of Calm2 mRNA was induced mainly in cells in the subependymal layer, although it appeared not to be expressed in ependymal cells. Gelsolin and MT-3 mRNAs were expressed at higher levels in the ependymal and subependymal layers in rats with TBI, whereas they were expressed only mildly in controls. MLP mRNA was more widely induced in the SVZ after TBI. The data suggest that TBI influences gene expression in the SVZ. The combination of cDNA microarray and *in situ* hybridization analyses is an efficient and powerful means to identify and characterize genes involved in the molecular mechanisms of pathophysiological changes in the SVZ after TBI.

TABLE 3. EXPRESSED SEQUENCE TAGS UP-REGULATED AFTER TBI

Accession number	*Fold change*	*Accession number*	*Fold change*	*Accession number*	*Fold change*
AA437717	2.7	AA760233	2.1	AA138313	2.0
AA656394	2.6	AA856261	2.1	AA607232	2.0
AI592184	2.3	AI120958	2.1	AA760121	2.0
AA073778	2.2	AI386049	2.1	AA763276	2.0
AA681081	2.2	AI464317	2.1	AI098372	2.0
AA874467	2.2	AI549639	2.1	AI098372	2.0
AA122581	2.1	W80245	2.1	AI450490	2.0
AA162693	2.1	AA015506	2.0	W29750	2.0
AA172493	2.1	AA047991	2.0	W30583	2.0
AA624579	2.1	AA106164	2.0		

GenBank accession numbers and fold change are listed.

TABLE 4. FUNCTIONAL CLASSIFICATION OF KNOWN GENES DOWN-REGULATED AFTER TBI

Gene name	*Accession number*	*Fold change*
Transcription factors		
Myelin basic protein expression factor 2, repressor	W83960	2.5
Zinc fingers and homeoboxes protein 1	AI120674	2.3
Zinc finger protein 118	AA242194	2.3
Zinc finger proliferation 1	AI159431	2.2
Zinc finger protein 37	AA388202	2.1
Zinc finger protein 90	AA517408	2.1
Zinc finger protein 46	AA269904	2.0
E4F transcription factor 1	AI324466	2.0
Receptors		
Stromal cell derived factor receptor 1	AA414208	2.7
Eph receptor A4	AI325333	2.4
Complement receptor 2	AA208784	2.3
Inositol 1,4,5-triphosphate receptor 1	AA444527	2.1
Very low density lipoprotein receptor	AA020307	2.1
Hyaluronan mediated motility receptor (RHAMM)	AA981371	2.0
Prolactin receptor related sequence 1	AA276003	2.0
Protein tyrosine phosphatase, receptor type, Z	AA968121	2.0
Intracellular signaling		
Protein phosphatase 3, catalytic subunit, alpha isoform	AA387372	3.1
CDC-like kinase	AA684191	2.9
Eph receptor A4	AI325333	2.4
Protein kinase C, beta	AA289586	2.4
Mitogen activated protein kinase kinase 4	AA414670	2.4
Protein kinase inhibitor, alpha	AI391322	2.3
Hect domain and RCC1 (CHC1)-like domain (RLD) 2	AA547373	2.2
V-ral simian leukemia viral oncogene homolog A (ras related)	AA212148	2.2
RAB10, member RAS oncogene family	AA619822	2.2
Rad and gem related GTP-binding protein	AI390842	2.1
Ras-GTPase-activating protein (GAP<120>) SH3-domain-binding protein 2	AA617613	2.1
Regulator of G-protein signaling 2	AA221794	2.0
CDC like kinase 4	AA444586	2.0
BMX non-receptor tyrosine kinase	AA138327	2.0
Protein tyrosine phosphatase, receptor type, Z	AA968121	2.0
Protein modification and maintenance		
Chaperonin subunit 8 (theta)	AW209866	2.1
Heat shock protein, 60 kDa	AA444576	2.0
Cathepsin S	AA146437	2.0
Cathepsin L	AA619763	2.0
Nucleic acid synthesis and modification		
Splicing factor, arginine/serine-rich 5 (SRp40, HRS)	AI120315	2.1
Channels and prions		
Inositol 1,4,5-triphosphate receptor 1	AA444527	2.1
Transporters and pumps		
ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	AA739359	3.1
ATPase, H+ transporting, lysosomal (vacuolar proton pump), alpha 70 kDa, isoform	AA068612	2.7
Secreted and extracellular		
SPARC-like 1 (mast9, hevin)	AA068603	2.5
Organelle		
ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	AA739359	3.1
Heterogeneous nuclear ribonucleoprotein A1	W11164	2.8
ATPase, H+ transporting, lysosomal (vacuolar proton pump), alpha 70 kDa, isoform	AA068612	2.7
Myelin basic protein expression factor 2, repressor	W83960	2.5
Zinc fingers and homeoboxes protein 1	AI20674	2.3
Zinc finger protein 118	AA242194	2.3
Zinc finger proliferation 1	AI159431	2.2