Serum after traumatic brain injury increases proliferation and supports expression of osteoblast markers in muscle cells

Cadosch, D; Toffoli, A M; Gautschi, O P; Frey, S P; Zellweger, R; Skirving, A P; Filgueira, L

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Abstract

BACKGROUND: Traumatic brain injury is associated with an increased rate of heterotopic ossification within skeletal muscle, possibly as a result of humoral factors. In this study, we investigated whether cells from skeletal muscle adopt an osteoblastic phenotype in response to serum from patients with traumatic brain injury.

METHODS: Serum was collected from thirteen patients with severe traumatic brain injury, fourteen patients with a long-bone fracture, and ten control subjects. Primary cultures of skeletal muscle cells isolated from patients undergoing orthopaedic surgery were performed and characterized with use of immunofluorescence microscopy, reverse transcription-polymerase chain reaction, and Western blot analysis. Proliferation and osteoblastic differentiation were assessed with use of commercial cell assays, Western blot analysis (for Osterix protein), and the Villanueva bone stain.

RESULTS: All serum-treated cell populations expressed the osteoblast marker Osterix after one week in culture. Cells treated with serum from all study groups in mineralization medium had increased alkaline phosphatase activity and mineralized nodules within the mesenchymal cell subpopulation after three weeks in culture. Serum from patients with traumatic brain injury induced a significant increase (p = 0.02) in the rate of proliferation of primary skeletal muscle cells (1.87 [95% confidence interval, 1.66 to 2.09]) compared with the rate induced by serum from patients with a fracture (1.42 [95% confidence interval, 1.21 to 1.58]) or by serum from controls (1.35 [95% confidence interval, 1.15 to 1.54]).

CONCLUSIONS: Human serum supports the osteoblastic differentiation of cells derived from human skeletal muscle, and serum from patients with severe traumatic brain injury accelerates proliferation of these cells. These findings suggest the early presence of humoral factors following traumatic brain injury that stimulate the expansion of mesenchymal cells and osteoprogenitors within skeletal muscle.
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Serum After Traumatic Brain Injury Increases Proliferation and Supports Expression of Osteoblast Markers in Muscle Cells

By Dieter Cadosch, MD, PhDc, Andrew M. Toffoli, MD, Oliver P. Gautschi, MD, Sönke P. Frey, MD, René Zellweger, MD, Allan P. Skirving, MD, and Luis Filgueira, MD

Investigation performed at the University of Western Australia, Crawley, and the Royal Perth Hospital, Perth, Western Australia, Australia

Background: Traumatic brain injury is associated with an increased rate of heterotopic ossification within skeletal muscle, possibly as a result of humoral factors. In this study, we investigated whether cells from skeletal muscle adopt an osteoblastic phenotype in response to serum from patients with traumatic brain injury.

Methods: Serum was collected from thirteen patients with severe traumatic brain injury, fourteen patients with a long-bone fracture, and ten control subjects. Primary cultures of skeletal muscle cells isolated from patients undergoing orthopaedic surgery were performed and characterized with use of immunofluorescence microscopy, reverse transcription-polymerase chain reaction, and Western blot analysis. Proliferation and osteoblastic differentiation were assessed with use of commercial cell assays, Western blot analysis (for Osterix protein), and the Villanueva bone stain.

Results: All serum-treated cell populations expressed the osteoblast marker Osterix after one week in culture. Cells treated with serum from all study groups in mineralization medium had increased alkaline phosphatase activity and mineralized nodules within the mesenchymal cell subpopulation after three weeks in culture. Serum from patients with traumatic brain injury induced a significant increase ($p = 0.02$) in the rate of proliferation of primary skeletal muscle cells (1.87 [95% confidence interval, 1.66 to 2.09]) compared with the rate induced by serum from patients with a fracture (1.42 [95% confidence interval, 1.21 to 1.58]) or by serum from controls (1.35 [95% confidence interval, 1.15 to 1.54]).

Conclusions: Human serum supports the osteoblastic differentiation of cells derived from human skeletal muscle, and serum from patients with severe traumatic brain injury accelerates proliferation of these cells. These findings suggest the early presence of humoral factors following traumatic brain injury that stimulate the expansion of mesenchymal cells and osteoprogenitors within skeletal muscle.

Clinical Relevance: A better understanding of the pathophysiological mechanisms that lead to the formation of heterotopic ossification in patients with a traumatic brain injury may be helpful in the prevention and treatment of this complication.

The incidence of heterotopic ossification after severe traumatic brain injury is between 11% and 25%.

Heterotopic ossification is characterized by the formation of lamellar bone at ectopic sites, such as skeletal muscle and connective tissue surrounding joints. Histologically and radiographically, heterotopic bone resembles normal mature bone and is distinguished from simple calcification by the nature of its osteoblastic capacity. It can cause pain and reduce the range of joint motion, and, at worst, it can result in complete ankylosis (in 10% to 16% of cases).

Heterotopic ossification requires stimulation of mesenchymal cell recruitment, proliferation, and differentiation, followed by osteoprogenitor maturation and osteoblast activation. Several groups of investigators, including ours, have suggested the importance of humoral factors in the cascade of events leading to heterotopic ossification on the basis of in vitro studies demonstrating the mitogenic and osteogenic effects of serum from subjects who had undergone trauma to the central nervous system. These investigators used models of osteoblasts, mesenchymal stem cells, and fibroblasts, but none

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reviewed the effect of serum from patients with traumatic brain injury on cells derived from skeletal muscle.

Heterotopic ossification occurs in skeletal muscle more frequently than it does in soft tissues, presumably because of skeletal muscle's blood supply and the presence of stem cells capable of osteogenic differentiation. Cells isolated from adult human skeletal muscle have been shown to be able to differentiate into osteoblastic cells in vitro and to form new bone when reimplanted into animals.

In the present in vitro study, we investigated the effects of serum from patients with traumatic brain injury on cells extracted from human skeletal muscle in an attempt to reveal the processes underlying heterotopic ossification.

**Materials and Methods**

**Patients and Specimen Collection**

Ethics approval was granted by the Royal Perth Hospital Human Research Ethics Committee, and all patients or their next of kin gave informed consent. Skeletal muscle was obtained from thirty-eight consecutive patients without known myopathy or a connective-tissue disorder who were undergoing orthopaedic surgery. Specimens were obtained from muscle in the lower limb (twenty-two samples) or the upper limb (fifteen samples). A sample of the rectus abdominis was excised from one patient. The mean age of the muscle donors was 37.6 years (range, eighteen to eighty years).

Serum was collected from twenty-seven consecutive individuals recruited from the Royal Perth Hospital, Perth, Western Australia. Thirteen patients with an isolated severe brain injury were treated at our level-I trauma center according to the Advanced Trauma Life Support guidelines. After primary diagnostic and therapeutic management, the patients were transferred to the intensive care unit. Fourteen patients with an isolated long-bone shaft fracture without a traumatic brain injury were also recruited. Ten healthy volunteers were recruited as control subjects. The overall mean age of the blood donors in the study was 33.8 years (range, eighteen to seventy-two years), and the mean ages did not differ significantly among the study groups (p > 0.05).

On admission, all patients with a traumatic brain injury were evaluated with the Glasgow Coma Scale and Abbreviated Injury Scale score. The type and extent of the hemorrhagic central nervous system lesion or diffuse cerebral edema were determined with computed axial tomography. The eligibility criteria for the patients with a traumatic brain injury were an isolated severe traumatic brain injury, a Glasgow Coma Scale score of ≤8 points, and an Abbreviated Injury Scale score of ≥2 points. The mean Glasgow Coma Scale score for these patients was 5.1 points (range, 3 to 8 points), and the mean Abbreviated Injury Scale score was 3.4 points (range, 2 to 5 points). The radiographic findings included subdural, epidural, and subarachnoid hemorrhage as well as shearing injuries and generalized brain edema.

All patients with an isolated bone fracture had sustained a fracture of the shaft of the femur (eight patients) or tibia (six patients). The long-bone fractures were imaged with conventional anteroposterior and lateral radiographs. Clinical and radiographic investigation revealed that none of the patients with a long-bone fracture had sustained a traumatic brain injury. Exclusion criteria included all forms of prior nervous system or bone-related disease, immunosuppression, rheumatoid arthritis, and diabetes as well as steroid or bisphosphonate therapy.

Blood samples were collected from all patients at four time points during the first week following the injury. The first sample was obtained as soon as possible after hospital admission but always within six hours after the injury. Subsequent samples were collected at twenty-four, seventy-two, and 168 hours after the injury. All specimens (except the first sample after the injury) were obtained at approximately 8:00 A.M. in order to reduce the circadian influence on biochemical markers and to maintain similar conditions in all groups. Samples were centrifuged at 1500 times gravity for ten minutes within thirty minutes after collection, and the resulting serum was stored at −80°C. The levels of alkaline phosphatase, adjusted calcium, inorganic phosphate, and parathyroid hormone were measured in all collected samples.

All patients were followed clinically for a minimum of twelve months after discharge. Clinical and radiographic assessments of heterotopic ossification (defined as ectopic bone in the muscle and connective tissue surrounding joints) were performed.

**Isolation and Culture of Human Skeletal Muscle Cells**

Isolation of cells from skeletal muscle was performed on the basis of previously described methods for obtaining primary skeletal muscle cell cultures. Briefly, muscle samples (approximately 1 cm³) were excised intraoperatively and transported in 0.9% saline solution. Muscle samples from different patients were not pooled. Samples were digested in 0.05% trypsin-EDTA (GIBCO Invitrogen, Auckland, New Zealand) for one hour, and the supernatant was centrifuged at 1200 rpm for eight minutes. The resultant cell pellet was redistributed into 25-cm² culture flasks (Sarstedt, Numbrecht, Germany) containing standard medium consisting of 5 mL of Dulbecco modified Eagle medium/Ham F-12 (DMEM/F12; GIBCO Invitrogen), 15% fetal calf serum (JRH Biosciences, a division of SAFC Biosciences, Lanoxa, Kansas), and 1% antibiotic-antimycotic liquid (10,000 U/mL of penicillin-G sodium, 10,000 µg/mL of streptomycin sulfate, and 25 µg/mL of amphotericin B; GIBCO Invitrogen). Cells were incubated at 37°C with 5% CO₂. The medium was changed weekly. When they approached confluence, cells were passed by trypsinization and redistribution into new flasks. Characterization was performed within the first three passages, and further experimentation was carried out within three passages after characterization. Cells were tested in multiple experiments with serum from different subjects.

**Immunohistochemical Characterization of Human Skeletal Muscle Cells**

Cells were fixed in 1% paraformaldehyde in 0.1M phosphate-buffered saline solution (GIBCO Invitrogen), pH 7.2, and...
permeabilized in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri). They were then exposed to primary monoclonal antibodies for alkaline phosphatase (hybridoma cell supernatant [B4-78], Mayo Clinic, Rochester, Minnesota), desmin (1:200, DE-U-10 clone; Sigma-Aldrich), and vimentin (1:200, VIM-13.2 clone; Sigma-Aldrich), followed by a donkey anti-mouse Alexa Fluor 488 secondary antibody (1:50; Molecular Probes, Eugene, Oregon). Counterstaining was performed with DAPI (4′,6-diamidino-2-phenylindole-dihydrochloride) (1:1000; F. Hoffmann-La Roche, Basel, Switzerland) and Alexa Flour 546 phallloidin (1:100; Molecular Probes). SYTO 25 green fluorescent nucleic acid stain (1:1000; Molecular Probes) was used as a nuclear stain in live cells.

**Isolation and Culture of Human Peripheral Blood Mononuclear Cells and T-Lymphocytes**

Human mononuclear cells and T-lymphocytes were obtained from peripheral blood mononuclear cells isolated from buffy coats of healthy blood donors (Australian Red Cross Blood Service [ARCBS], Perth, Western Australia, Australia) through Ficoll-gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). The isolated peripheral blood mononuclear cells were cultured (37°C, humidified, 5% CO₂) in 25-cm² tissue-culture flasks (Sarstedt) in RPMI-1640 GlutaMAX medium (GIBCO Invitrogen), supplemented with 5% human serum (ARCBS) and 1% antibiotic-antimycotic liquid. After one hour in culture, the nonadherent peripheral blood mononuclear cells (95% of which were T-lymphocytes) were collected and washed twice with phosphate-buffered saline solution before being processed further as described below. The cultures of adherent cells (95% of which were mononuclear cells) were gently washed three times with 10 mL of phosphate-buffered saline solution at room temperature and replenished with 5 mL of fresh RPMI-1640 GlutaMAX medium supplemented with 1% antibiotic-antimycotic liquid. Thereafter, the cells were gently scraped from the flasks and used as described below. Ethical approval for using human blood cells for this study was granted by the Ethics Committee of the University of Western Australia and the ARCBS.

**Assessment of Proliferation of Human Skeletal Muscle Cells**

The colorimetric CellTiter 96 MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay (Promega, Madison, Wisconsin) was used to assess the effect of serum collected from peripheral blood mononuclear cells isolated from buffy coats of healthy blood donors (Australian Red Cross Blood Service [ARCBS], Perth, Western Australia, Australia) through Ficoll-gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) at an absorbance of 405 nm. Data were subsequently expressed as the percentage of absorbance relative to the absorbance of the cells isolated from the same individual and incubated without serum during the same period of time.

**Western Blot Analysis**

Western blot analysis was used to detect cell expression of Osterix protein. Near confluence, the standard medium was replaced with serum-free medium for twenty-four hours, and then 5% serum from the traumatic brain injury or control group was added. After one week, cells were lysed with a whole-cell lysate buffer. A 12% sodium dodecyl sulfate polyacrylamide gel was used for two-dimensional protein separation. Twenty-five microliters of protein was loaded into each well, and electrophoresis was performed at 100 to 150 V. Proteins were transferred to nitrocellulose membranes (Amersham Biosciences), and excess antigens were blocked with 5% milk for thirty minutes. Membranes were incubated with 1:500 primary antibody (goat polyclonal anti-Osterix [Santa Cruz Biotechnology, Santa Cruz, California]; and mouse monoclonal anti-actin [Sigma-Aldrich]) overnight at 4°C. For Osterix, a biotinylated donkey anti-goat secondary antibody (1:1000; Santa Cruz Biotechnology) was used for one hour at room temperature. Membranes were then incubated with a horseradish peroxidase conjugated goat anti-rabbit IgG (1:10,000; Dako, Glostrup, Denmark) for one hour at room temperature. SuperSignal West Pico reagents (Pierce Biotechnology, Rockford, Illinois) were added to the membrane for five minutes, and signals were visualized on a Kodak 2000MM Imaging System (Kodak, Rochester, New York). After stripping, β-actin was used as an internal positive control. Human fetal osteoblasts (hFOB 1.19; American Type Culture Collection, Manassas, Virginia) cultured in standard medium were used to ensure antibody specificity.

**Mineralization and Alkaline Phosphatase Activity**

Cells were suspended in six-well plates (Sarstedt) containing standard medium. At confluence, the standard medium was replaced with serum-free medium. After twenty-four hours, a mineralization medium (DMEM/F12, 1% antibiotic-antimycotic liquid, 2mM β-glycerophosphate, and 12.5 μg/mL of ascorbic acid) and 5% serum from the traumatic brain injury or
control group were added. The medium was changed every five days, and the supernatant was collected for measurement of alkaline phosphatase activity. Supernatants were analyzed with the Roche Hitachi 917 analyzer (Roche Diagnostics, Rotkreuz, Switzerland), which employs a spectrophotometer to determine alkaline phosphatase activity on the basis of the production of p-nitrophenol. After three weeks, cells were fixed in 70% ethanol and the Villanueva osteochrome bone stain (Polysciences Europe, Eppelheim, Germany) was performed in situ. One milliliter of reagent was added to each well, and, after ninety minutes, the cells were washed with distilled water and 70% ethanol until the wash solution was colorless. The nodules in each well were counted and averaged over three plates.

**Statistical Analysis**

A two-way repeated-measures analysis of variance was used to test mean differences between the study groups in terms of cell proliferation rates and in terms of serum marker concentrations. Independent-samples t tests were used to determine specific differences according to group and the time point after injury. Pearson correlations (bivariate) were used to analyze the continuous variables, including patient age, proliferation rate, Glasgow Coma Scale score, Abbreviated Injury Scale score, and serum marker concentrations. A p value of <0.05 was considered significant.

**Source of Funding**

There was no external funding source for this study.
Results

Biochemical Markers and Clinical Assessment of Heterotopic Ossification

There were no significant differences in serum alkaline phosphatase, phosphate, or parathyroid hormone concentrations between the groups at any time point (p > 0.05). The serum calcium levels in the patients with a traumatic brain injury were significantly lower than those in the patients with an isolated long-bone fracture or the control patients across all time points (p < 0.05). Radiographic evidence of heterotopic ossification was found in four of the thirteen patients with an isolated traumatic brain injury. None of the patients with an isolated long-bone fracture revealed evidence of heterotopic ossification on clinical or radiographic follow-up.

Skeletal Muscle Cell Characterization

The thirty-eight primary skeletal muscle cell cultures were tested for expression of myogenic genes with use of reverse transcription-polymerase chain reaction. All cell cultures expressed myosin heavy chain IIa (MyHCIIa). Additionally, morphological methods were used for further cell characterization. Cell populations consisted primarily of two cellular morphologies: elongated, multinucleated cells (skeletal muscle fibers) and spindle-shaped cells (precursor cells). Immunohistochemical staining was used to visualize specific markers within the skeletal muscle cell population. The myogenic tendency of the cell populations was demonstrated by desmin staining of elongated cells containing multiple nuclei, consistent with myotubes. The stromal nature of the spindle-shaped cell population was exemplified by the high degree of positive staining for vimentin. Some spindle-shaped cells constitutively stained positive for alkaline phosphatase (Fig. 1).

Serum from Patients with Severe Traumatic Brain Injury Increases Muscle Cell Proliferation

One of the aims of this study was to investigate the effects of serum from patients with traumatic brain injury on the proliferation rates of cells extracted from human skeletal muscle. The first step was to evaluate whether the age of the muscle donor influenced cell proliferation. For that purpose, human skeletal muscle cells isolated from ten donors with a wide range of ages (eighteen to eighty years) were exposed to the same serum collected from either patients with traumatic brain injury or controls (three samples per group). With the numbers studied, the investigations revealed no significant age-dependent differences in the proliferation rates of skeletal muscle cells exposed to serum in vitro (p > 0.05). Consequently, the range of ages of the muscle donors was not restricted. The time after injury did not affect muscle cell proliferation within the first three days (p > 0.05). However, analysis showed a maximum of activity that was slightly, but
not significantly, greater than the earlier level at twenty-four hours after injury, with the activity remaining at the same level for three days before declining one week after the injury. As a result, the serum samples collected at twenty-four hours after the injury were chosen for all subsequent experiments. All thirty-seven serum samples were tested for their proliferation potential in five primary muscle cell cultures as well as in five cultures of human T-lymphocytes and monocytic cells collected from five different donors. Repeated-measures analysis of variance showed no significant difference in proliferation between the cells from different donors cultured in the presence of the same serum ($p = 0.42$). Each of the thirty-eight muscle specimens was exposed to each type of serum.

The mean proliferation rate of the skeletal muscle cells exposed to serum from patients with severe traumatic brain injury (1.87 [95% confidence interval, 1.66 to 2.09]) was significantly greater ($p = 0.02$) than the proliferation rates of cells exposed to serum from patients with a long-bone fracture (1.42 [95% confidence interval, 1.21 to 1.58]) and from control subjects (1.35 [95% confidence interval, 1.15 to 1.54]) (Fig. 2). The serum from patients with a traumatic brain injury did not increase the proliferation rates of human T-lymphocytes or monocytic cells when compared with the serum from patients with an isolated fracture or that from controls ($p > 0.05$).

**Serum Supports an Increase in Alkaline Phosphatase Activity**

Alkaline phosphatase activity was measured in the culture supernatant of five primary muscle cell lines cultured in the presence or absence of normal serum and traumatic brain injury-group serum that had induced the highest rate of muscle cell proliferation. Both the cell populations treated with serum from the traumatic brain injury group and those treated with the control serum exhibited an increase in alkaline phosphatase activity over the duration of the experiments. A significant difference in alkaline phosphatase activity was found between both study groups and the serum-free negative control ($p < 0.05$), but there was no significant difference between the serum-treated groups ($p = 0.20$).
Serum Supports Osterix Protein Expression

Osterix expression was measured as a late osteoblastic differentiation marker. For that purpose, five different primary muscle cell lines were cultured in the presence or absence of at least three different samples of serum from both the traumatic brain injury group and the control group. Western blot analysis revealed a band at the expected molecular weight (45 kDa) for the Osterix protein in the serum-treated groups but not in the serum-free negative controls, indicating the induction of Osterix expression by human sera from both groups. Lanes containing protein from hFOB 1.19 cells cultured in standard medium containing 15% fetal calf serum showed the same band (Fig. 3).

Serum Supports Mineralization of Skeletal Muscle Cells

Formation of bone nodules and production of calcified extracellular matrix was tested with five primary muscle cell cultures in the presence of the traumatic brain injury-group serum that had induced the highest rate of muscle cell proliferation and in the presence of control serum. Villanueva staining of the skeletal muscle cells after three weeks of culture in a mineralization medium showed frequent condensations of spindle-shaped cells and small numbers of positively stained nodules arising from spindle-shaped cells in the serum-treated groups; these findings were not present in the untreated negative controls (Fig. 4). At least two of three plates for both serum study groups contained Villanueva-positive nodules. The mean numbers of Villanueva-positive nodules (and the standard deviation) per well were 1.7 ± 2.1 and 1 ± 1 in the traumatic brain injury group and the normal control group, respectively. There was no significant difference in the number of nodules between the two serum groups (p > 0.05).

Discussion

We previously demonstrated that patients with a traumatic brain injury and a femoral shaft fracture have enhanced fracture-healing, with a twofold shorter time to union and increased callus formation compared with patients with a femoral fracture but no traumatic brain injury. Proteomic investigations have demonstrated a change in the profile of serum and cerebrospinal fluid proteins in response to traumatic brain injury that is potentially linked to the osteogenic influence that traumatic brain injury has on fracture-healing. Additionally, serum from subjects with a traumatic injury to the central nervous system has been shown to increase the proliferation and alkaline phosphatase expression of mesenchymal and osteoblastic cells. Bidner et al. showed that sera from patients with a traumatic brain injury increased fetal rat calvarial cell proliferation compared with that induced by sera from a group without a traumatic brain injury. Boes et al. found that pooled sera from rats with a traumatic brain injury and a fracture increased proliferation of mesenchymal stem cells, but not fibroblastic or osteoblastic cell lines, compared with that induced by sera from rats with a fracture alone. Sera from rats with a traumatic brain injury have also been shown to increase alkaline phosphatase activity in rat bone-marrow stromal cells. Taken together, these results strongly support the concept that osteoinductive factors are released by the injured brain into the systemic blood circulation and subsequently act peripherally on the affected soft tissues. The same humoral factors may also be implicated in the development of heterotopic ossification within skeletal muscle following a traumatic brain injury. It has been suggested that the consolidated callus that has been observed in patients with traumatic brain injury is a form of local heterotopic bone formation. This suggestion has been supported by the histological finding that callus at the sites of fractures in patients with a traumatic brain injury more closely resembles heterotopic bone than normal fracture callus.

The osteogenic capability of skeletal muscle cell populations was suggested by the constitutive high levels of alkaline phosphatase at the protein and functional level. Alkaline phosphatase is thought to be required for the release of inorganic phosphate into the osseous matrix, but it is also a relatively early marker of osteoblastic lineage that can be used to assess the osteogenic potential of clonal populations of osteoprogenitors. Interestingly, it has been detected in intramuscular connective tissue. During extended culture, the alkaline phosphatase activity within the serum-treated muscle cell populations increased significantly in our study, emphasizing the growth-promoting effects of serum on cells expressing functional alkaline phosphatase. Kurer et al. showed that serum both from patients with a spinal cord injury and control volunteers increased the alkaline phosphatase activity in cells extracted from human femora. Klein et al. also demonstrated an increase in alkaline phosphatase activity in rat bone-marrow stromal cells exposed to serum obtained from rats after traumatic brain injury.

Untreated skeletal muscle cell populations in our study did not show evidence of Osterix protein expression, which is consistent with an immature state of osteoblastic differentiation. Osterix is specific to the osteoblastic lineage, being found only within the bone matrix, the endosteum, and the periosteum postnatally. Mice lacking Osterix have a complete absence of osteoblasts, a finding that emphasizes its importance in the commitment of osteoprogenitor cells to become mature osteoblasts. Skeletal muscle cells cultured with serum from both experimental groups in our study showed evidence of induction of Osterix protein and generated mineralized nodules. These findings imply that sera from normal individuals and those with traumatic brain injury contain factors capable of supporting osteoblastic maturation and mineralization by putative skeletal muscle osteoprogenitors in vitro.

Mineralized nodules arose from vimentin-positive spindle-shaped cells, as opposed to cells with the myotube morphology, implying that cells involved in ossification within skeletal muscle cells in vitro might lie external to the myofiber. The expression of vimentin is associated with the mesenchymal phenotype, including fibroblasts and skeletal muscle stem cells. Some of the spindle-shaped cells present in skeletal muscle have been linked to mesenchymal stem cells,
bone-marrow stromal cells, and pericytes because of their similar morphological, immunohistochemical, and mRNA profiles and their capacity for multilineage differentiation. The involvement of the skeletal muscle interstitium in heterotopic ossification has been suspected for decades. The central role of mesenchymal cells from skeletal muscle connective tissue in the development of heterotopic ossification following trauma to the central nervous system is supported by histological analysis of osseous deposits.

The results of this study indicate a relationship between severe traumatic brain injury and serum-induced proliferation of skeletal muscle cells. Widespread DNA synthesis throughout the proliferated cell cultures was detected predominantly in isolated nuclei or aggregations of nuclei, a finding consistent with bright-field observations of increased fibroblastic cell numbers and the formation of cellular condensations. It is plausible that the additional proliferative effect of serum factors present after severe traumatic brain injury on skeletal muscle mesenchymal and early osteoblastic cells shifts the balance toward the development of bone in a tissue in which bone is not normally found. This proliferative effect may be essential for the development of heterotopic ossification in these patients because it produces an excess of cells with osteogenic potential within soft tissues. The finding that serum taken at six, twenty-four, or seventy-two hours after traumatic brain injury induced similar mean proliferation rates suggests that the processes underlying the observed serum-mediated proliferation begin at least as early as six hours after injury and continue at a relatively constant rate throughout the first three days after injury.

**References**


