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Transforming Growth Factor-β2 and TGF-β3 Regulate Fetal Rat Cranial Suture Morphogenesis by Regulating Rates of Cell Proliferation and Apoptosis

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ABSTRACT **Cranial vault sutures are the major intramembranous bone growth sites during rapid expansion of the neurocranium. To function as bone growth sites, sutures need to remain patent, while allowing rapid bone formation at the edges of the bone fronts. Premature osseous obliteration of sutures (craniosynostosis) by fusion of bone fronts across the suture site prevents further bone formation at this site, often leading to severe facial dysmorphology. Although several growth factor receptor and transcription factor mutations have been implicated in craniosynostosis, the underlying mechanisms leading to sutural obliteration remain unclear. Previous studies have shown that dura secreted soluble factors responsible for maintaining suture patency and that suture fusion observed in the absence of dura was preceded by elevated levels of DNA synthesis and collagen production in the suture region. The use of neutralizing antibodies in a fetal calvarial culture model further demonstrated that removal of transforming growth factor (TGF) -β3 activity induced premature sutural obliteration, whereas removal of TGF-β2 activity prevented sutural obliteration. Data presented here demonstrate that suture obliteration induced by removal of TGF-β3 activity was preceded by elevated levels of DNA synthesis, similar to that seen upon removal of the dura. Addition of exogenous TGF-β3 to calvaria cultured without dura both prevented suture obliteration and reduced DNA synthesis to levels comparable to those seen with intact dura. Addition of exogenous TGF-β2 to calvarial cultures induced sutural fusion accompanied by elevated levels of cell proliferation. However, sutures rescued from obliteration by removal of TGF-β2 activity did not have decreased levels of cell proliferation, but rather appeared to be due to inhibited differentiation. In all calvaria in which sutures remained patent in culture, numbers of apoptotic cells were high within the suture, whereas in sutures destined to fuse, numbers of apoptotic cells were low. Results indicate that one of the critical regulators of suture patency is cell number. Alterations in cell number can trigger premature differentiation of cells, resulting in sutural obliteration. Furthermore, a complex interplay between closely related molecules is required to maintain cranial vault sutures in an unossified state, while allowing new bone to be formed at the edges of the bone fronts.** © 2000 Wiley-Liss, Inc.

Key words: craniosynostosis; cranial vault; TGF-β2; TGF-β3; intramembranous bone growth; cell proliferation; apoptosis; rat

INTRODUCTION

Initial bone formation in the craniofacial region proceeds by expansive growth around several small, separate bones. Although bones thicken by accretional growth ectocranially (periosteally), continued expansive intramembranous bone growth depends on the formation and maintenance of sutures when craniofacial bones approximate one another. Sutures are the major sites of bone growth during craniofacial development (Baer, 1954). Craniosynostosis or premature obliteration of sutures leads to abnormal compensatory morphogenesis throughout the head (Enlow, 1989; Cohen, 1993).

Mutations in genes for fibroblast growth factor receptors 1, 2, and 3 (*FGFR1*, *FGFR2*, and *FGFR3*) are associated with human conditions involving craniosynostosis (Jabs et al., 1993, 1994; Muenke et al., 1994, 1997; Reardon et al., 1994; Wilkie et al., 1995; Bellus et al., 1996; Meyers et al., 1996), as are mutations in *MSX2* (Jabs et al., 1993; Ma et al., 1996) and *TWIST* (el Ghouzzi et al., 1997; Howard et al., 1997) genes. These are activating mutations, either through constitutive activation pathways (Galvin et al., 1996), negative regulation of bone growth (Deng et al., 1996), repression of developmental genes (Naski et al., 1998), or increased affinity for ligand (Anderson et al., 1998). Many of

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these gene products interact to regulate expression of one another (Rice et al., 1997, 1999; Kim et al., 1998) through tissue interactions between dura mater, bone fronts, and sutures (Opperman et al., 1993, 1995, 1996; Iseki et al., 1997; Kim et al., 1998). Significantly, although expression levels of members of the TGF-β family were found to be altered during normal and abnormal suture closure (Lin et al., 1997; Opperman et al., 1997; Roth et al., 1997a,b), no mutations in TGF-βs or their receptors have been reported to be associated with human suture pathology. Interestingly though, removal of TGF-β3 activity from fetal rat calvaria in vitro induced suture obliteration, whereas removal of TGF-β2 activity rescued sutures from obliteration (Opperman et al., 1999).

The mechanisms by which growth factors maintain sutures in their unobliterated state, yet enable them to produce new bony tissue, essential to their function as bone growth centers, are beginning to be elucidated. An initial clue to possible mechanism was the finding of elevated cell proliferation followed by increased collagen production within sutures destined to fuse in the absence of dura mater (Opperman et al., 1998). Furthermore, addition of FGF-2 and FGF-4 to cultured fetal mouse calvaria induced premature suture fusion, associated with elevated levels of cell proliferation, as did overexpression of MSX2 (Kim et al., 1998; Liu et al., 1999). However, studies on human craniosynostotic tissue fail to show elevated levels of cell proliferation but instead show increased cell differentiation and bone formation (De Pollak et al., 1996; Lomri et al., 1998; Fragale et al., 1999). Recently, Rice et al. (1999) have shown that apoptosis occurs in sutures during normal suture morphogenesis, proposing that apoptosis is a normal part of suture development. Any perturbation of normal levels of apoptosis that resulted in abnormal numbers of cells could contribute either to the premature obliteration of sutures or to failure of suture formation (Rice et al., 1999). Although alterations in numbers of apoptotic cells in response to growth factors has been reported in limb bud morphogenesis (Ganan et al., 1996; Macias et al., 1996), it has yet to be demonstrated during suture morphogenesis.

The working hypothesis based on these observations is that altering the concentration balance between growth factors, or altering tissue responsiveness to growth factors, will pathologically perturb suture patency. The purpose of this study was to test the hypothesis that addition of TGF-β3 to calvaria in vitro would rescue coronal sutures from obliteration accompanied by decreased rates of cell proliferation and increased apoptosis. Addition of TGF-β2 would induce suture fusion accompanied by increased proliferation and decreased apoptosis. Indices of cell proliferation, differentiation, and apoptosis were measured to elucidate the mechanism of action of these growth factors in regulating suture patency.

RESULTS

Rescue of Sutures From Osseous Obliteration by TGF-β3

Removal of TGF-β3 activity from cultures of F19 rat calvaria with intact dura mater resulted in coronal suture obliteration (Opperman et al., 1999), similar to that seen upon removal of dura mater (Opperman et al., 1998). Therefore, experiments were done to test whether addition of TGF-β3 to F19 rat calvaria cultured without dura mater could rescue sutures from obliteration. Although TGF-β3 concentrations of 0.03 ng/ml and 0.3 ng/ml prevent sutures from becoming obliterated after 5 days in culture, these sutures were markedly narrowed compared with open sutures. Sutures showed some intermittent regions of fusion of the frontal and parietal bones across the suture site (Fig. 1A). In the presence of 3 ng/ml TGF-β3, sutures were completely rescued from obliteration, with the two bone fronts remaining separated by a highly cellular suture matrix after 5 days in culture (Fig. 1B). When 30 ng/ml TGF-β3 was added to cultures, obliteration of the sutures was again noted after 5 days in culture (Fig. 1C). Because fusion across the sutures occurred intermittently, the degree of fusion along a series of sections was scored for each TGF-β3 concentration. As summarized in Table 1, sutures cultured for 5 days in the absence of dura mater and TGF-β3 were completely fused (F), whereas at 0.03 and 0.3 ng/ml TGF-β3, sutures were markedly narrowed (N). However, when 3 ng/ml TGF-β3 was added, sutures remained open (O). Complete fusion of sutures was again seen with addition of 30 ng/ml TGF-β3.

Suture Obliteration Induced by TGF-β2

Previous experiments showed that removal of TGF-β2 activity from coronal sutures of F19 rat calvaria cultured without the dura mater resulted in rescue of these sutures from obliteration (Opperman et al., 1999). To test whether TGF-β2 could induce fusion of normally nonfusing sutures from calvaria cultured with intact dura mater, TGF-β2 was added to these cultures. In the presence of 3 ng/ml TGF-β2, coronal sutures underwent osseous obliteration (Fig. 1D; Table 1) similar to that seen previously in the absence of dura mater (Opperman et al., 1995, 1998).

Effect of TGF-β3 on Cell Proliferation

Elevated levels of cell proliferation preceded fusion of sutures induced by removal of dura mater (Opperman et al., 1998). To examine whether similar increased cell proliferation preceded suture fusion associated with removal of TGF-β3 activity, tritiated thymidine uptake was measured in coronal sutures and calvarial bone cultured in the presence and absence of TGF-β3 neutralizing antibodies. Removal of TGF-β3 activity led to significantly ($P < 0.0001$) elevated levels of thymidine uptake in coronal sutures cultured for 3 days with intact dura mater (Fig. 2A), although levels were not as

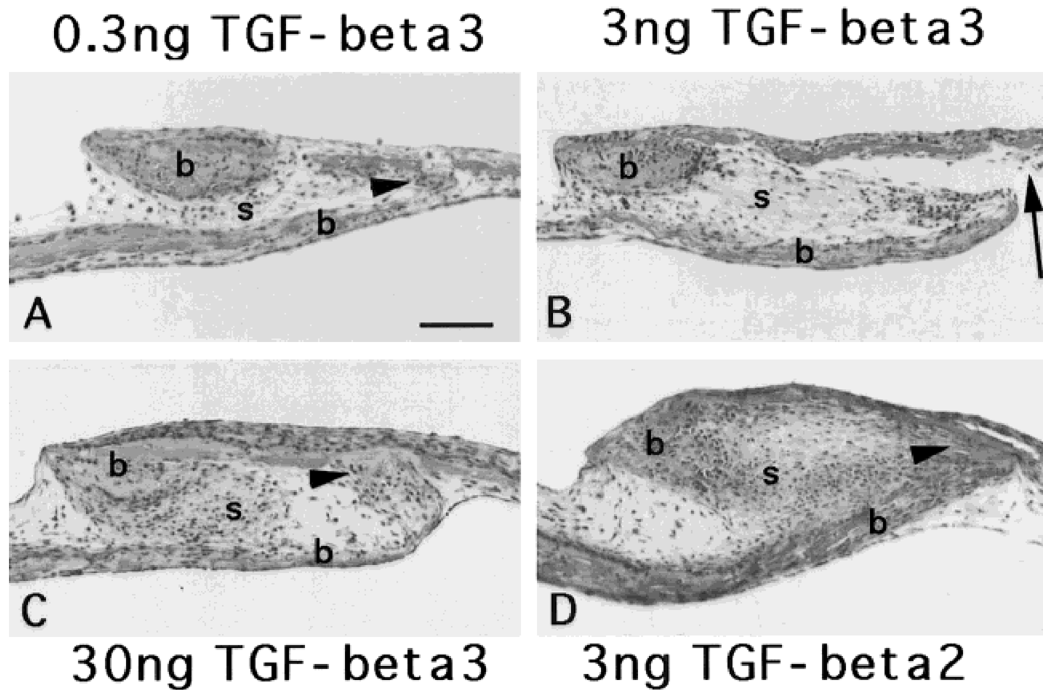

Fig. 1. Photomicrographs showing hematoxylin and eosin stained parasagittal sections through coronal sutures of F19 rat calvaria cultured for 5 days without dura mater and with 0.3 ng/ml (**A**), 3 ng/ml (**B**), or 30 ng/ml (**C**) transforming growth factor (TGF)-β3, or with dura mater and 3 ng/ml TGF-β2 (**D**). Note overlapping frontal and parietal bones (b) with intervening suture (s) in all sections. After 5 days in culture, bone fronts fused across the suture in the absence of dura mater (Opperman et al., 1995). No rescue of sutures was seen with either 0.3 or 30 ng/ml TGF-β3, and bone fronts fused across the suture (arrowheads in A,C). However, 3 ng/ml TGF-β3 rescued sutures from obliteration, with suture tissue remaining between the edges of the overlapping bone fronts (arrow in B). Sutures cultured in the presence of dura mater remained patent after 5 days in culture (Opperman et al., 1995). These sutures became obliterated with the addition of 3 ng/ml TGF-β2 (arrowhead in D). Scale bar = 200 μm in A (applies to A–D).

TABLE 1. Scoring of Suture Patency in the Presence of TGF-β2 or Increasing Concentrations of TGF-β3[a]

Parameter	Dura	3 days	5 days
TGF-β3 (ng/ml)			
0	—	F (6)	F (5)
0.03	—	O (5)	N (6)
0.3	—	O (5)	N (4)
3.0	—	N (6)	O (5)
30	—	F (2)	F (2)
TGF-β2 (ng/ml)			
0	+	O (4)	O (4)
3.0	+	O (4)	F (4)

[a]O, open suture; N, narrow suture but not fused; F, fused suture. Number of sutures is given in parentheses.

high as those seen in the absence of dura mater. Levels remained elevated at 4 and 5 days of culture when compared with calvaria cultured with intact dura; however, these differences were not significant. Although addition of neutralizing antibodies did not alter thymidine uptake at 3 days when added to calvaria cultured without dura mater, levels in these tissues remained elevated at 4 days, when thymidine uptake in sutures cultured without dura were beginning to decline. After 5 days in culture, no significant differences between the groups were noted. Elevated levels of thymidine uptake were also noted in the calvarial bones, both in the absence of dura mater and absence of TGF-β3 activity (Fig. 2B).

Addition of increasing concentrations of TGF-β3 (from 0–0.3 ng/ml) to F19 calvaria cultured without dura mater resulted in decreased amounts of DNA synthesis at 3 days in culture compared with calvaria cultured in the absence of dura mater (Fig. 3). This produced a sum of squares by regression analysis of 9.537 with a significance of $P < 0.008$. Interestingly, the regression analysis predicted that the optimal dose of TGF-β3 would be 2.5 ng/ml, because by 3 ng/ml, the amount of DNA synthesis was once again beginning to rise. This is supported by the fact that at 30 ng/ml TGF-β3 failed to prevent obliteration of sutures and elevated levels of DNA synthesis similar to that seen in the absence of dura mater were noted (data not shown). By 4 days in culture, the dose dependency has disappeared, with all sutures cultured in the presence of TGF-β3, regardless of concentration showing reduced levels of DNA synthesis when compared with controls (Fig. 3). By 5 days in culture, control levels are decreased and are no different to levels seen in experimental explants.

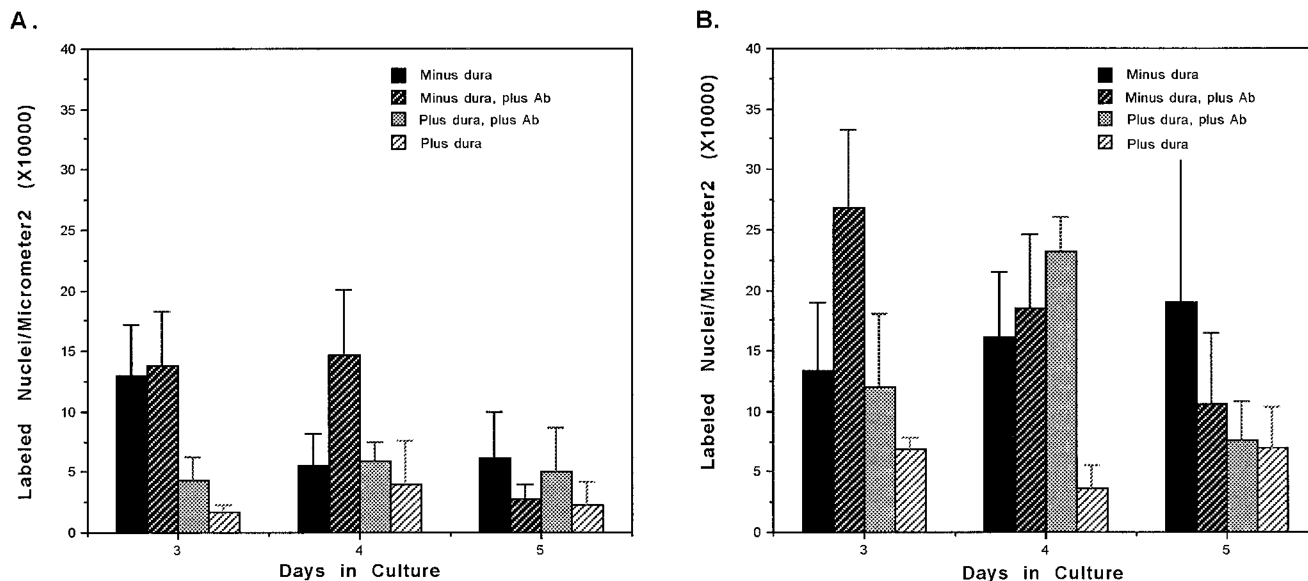

Fig. 2. Tritiated thymidine uptake in coronal sutures (**A**) and frontal and parietal bones (**B**) of F19 rat calvaria cultured in the presence (plus Ab) and absence of neutralizing antibodies to transforming growth factor-β3 established by autoradiography. Thymidine uptake in sutures cultured with dura mater and the presence of Ab was significantly elevated ($P < 0.0001$) when compared with sutures cultured with dura mater but without Ab after 3 days in culture. No significant differences were noted after 4 and 5 days in culture in either bones or sutures or between tissues cultured without dura mater.

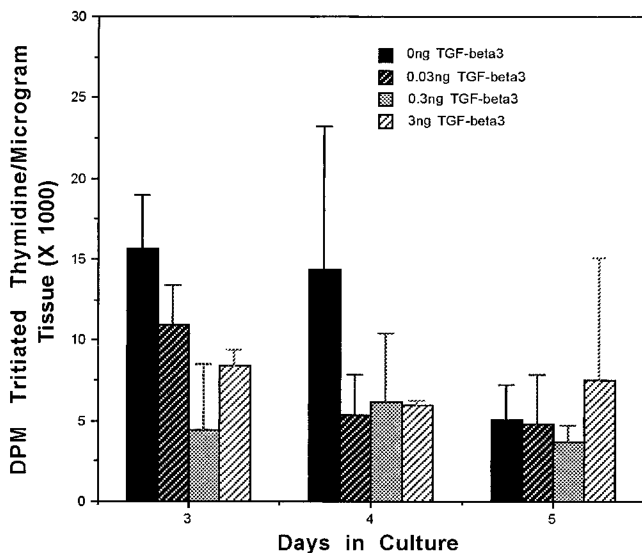

Fig. 3. Tritiated thymidine uptake in coronal sutures of F19 rat calvaria cultured without dura mater and with increasing amounts of transforming growth factor (TGF)-β3 measured by scintillation counting. Significantly ($P < 0.009$) decreased amounts of DNA synthesis were noted after 3 and 4 days in culture with addition of TGF-β3 when compared with sutures cultured in the absence of TGF-β3. After 5 days in culture, DNA synthesis had declined in all tissues, and no differences between tissues were noted.

Effect of TGF-β2 on Cell Proliferation

Removal of dura mater (Opperman et al., 1995, 1998) or removal of TGF-β3 activity (Opperman et al., 1999) led to suture obliteration. In both cases, elevated levels of thymidine uptake, which is an index of cell proliferation, preceded suture obliteration. To examine whether sutural obliteration induced by TGF-β2 is accompanied by elevated levels of cell proliferation, calvaria cultured with TGF-β2 were pulsed with tritiated thymidine and examined after 3, 4, and 5 days in culture. Addition of TGF-β2 led to significantly ($P < 0.001$) elevated levels of cell proliferation both in coronal sutures and in the calvarial bones at 3 days in culture (Fig. 4). Although thymidine uptake remained elevated at 4 days, the levels were greatly reduced from those seen at 3 days. These levels declined by 5 days in culture to levels similar to those seen in tissues cultured without TGF-β2. In contrast to this finding, when TGF-β2 neutralizing antibodies were added to F19 calvarial cultures, sutures were rescued from obliteration; however, the levels of DNA synthesis in the coronal sutures remained unaltered, both in the presence and absence of the dura mater (Fig. 5A). Similarly, levels of cell proliferation in the calvarial bones were also unaltered (Fig. 5B).

Effect of TGF-β3 on Apoptosis in Rat Coronal Sutures

To examine whether rescue of sutures from osseous obliteration by addition of TGF-β3 was accompanied by cell death, apoptosis assays were conducted on histologic sections from coronal sutures of calvaria cultured without dura mater and treated with either 0 ng or 3 ng/ml TGF-β3. Few apoptotic cells were present in the sutures of calvaria not treated with TGF-β3 at both 3 and 5 days in culture (Fig. 6A,C). Increased levels of

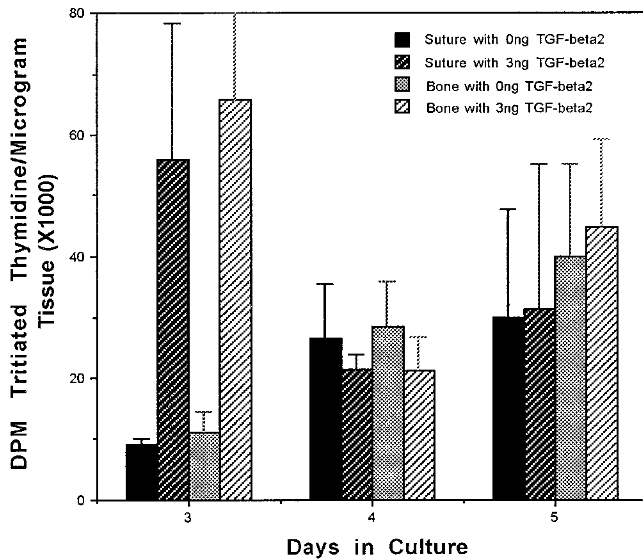

Fig. 4. Tritiated thymidine uptake in coronal sutures and frontal and parietal bones of F19 rat calvaria cultured without dura mater and with or without 3 ng/ml transforming growth factor (TGF) -β2 measured by scintillation counting. Both sutures and bones of calvaria cultured in the presence of TGF-β2 showed significantly elevated levels of DNA synthesis ($P < 0.0001$) after 3 days in culture when compared with sutures and bones cultured without TGF-β2. After 4 and 5 days in cultures, DNA synthesis in these tissues declined to levels similar to those of control tissues.

apoptotic cells were observed in the sutural tissue within the overlapping bone fronts, after addition of TGF-β3. Apoptotic nuclei were also seen at the leading edges of both frontal and parietal bone fronts (Fig. 6B,D). Apoptotic cells were seen in the periosteum of all calvaria examined (arrows) and in the osteoblasts of the calvarial bones away from the suture region (not shown).

Effect of TGF-β2 on Apoptosis in Rat Coronal Sutures

Because elevated levels of apoptotic cells was associated with sutures rescued from obliteration by TGF-β3, it was examined whether suture obliteration induced by addition of TGF-β2 was accompanied by altered levels of apoptotic cells. Sections through coronal sutures of calvaria cultured with intact dura mater and treated with either 0 or 3 ng/ml TGF-β2 were tested. Sutures from calvaria with intact dura mater and no TGF-β2 showed high levels of apoptotic cells both along the bone fronts and within the suture tissue (Fig. 7A,C), similar to that seen with the addition of 3 ng/ml TGF-β3 to calvaria cultured without dura mater (Fig. 6B). When 3 ng/ml TGF-β2 was added to cultures, almost no apoptotic cells were seen after 3 days in culture, either in the bone fronts or in the suture itself (Fig. 7B). After 5 days in culture, apoptotic cells could be seen along the bone fronts, but not in the remnants of the suture matrix (Fig. 7D).

Effect of TGF-β3 Administration on TGF-β2 Immunoreactivity

To test whether TGF-β3 rescued sutures from obliteration by decreasing TGF-β2 present in tissues, immunohistochemistry was used to assess any changes in TGF-β2 immunoreactivity in response to increasing concentrations of TGF-β3. There were no changes in the distribution, timing of appearance, or intensity of TGF-β2 immunoreactivity in the bone matrix, suture matrix or cells (data not shown), with either 3 or 30 ng/ml TGF-β3. Similar low intensity of TGF-β2 immunoreactivity was seen in the bone-lining cells and suture cells of calvaria cultured in the presence or absence of TGF-β3, as was reported previously for tissues cultured in the presence and absence of dura mater (Opperman et al., 1997). Controls in which no primary antibodies were added showed no immunoreactivity in any tissues (not shown).

DISCUSSION

Results from this study demonstrated that TGF-β2 and TGF-β3 play important and antagonistic roles in regulating rat coronal suture patency. Previous studies had shown that coronal sutures from cultured fetal rat calvaria with intact dura mater could be maintained in culture for up to 2 weeks. Removal of the dura mater resulted in sutural obliteration after 5 days in culture (Opperman et al., 1995). By using this model system, TGF-β2 or TGF-β3 were added to the culture system to examine the mechanisms by which these growth factors modulate suture patency.

Addition of TGF-β3 to cultured fetal rat calvaria rescued coronal sutures from obliteration in a dose-dependent manner. Interestingly, when TGF-β3 concentrations became extremely elevated, sutural obliteration was again noted, indicating that the critical concentration of TGF-β3 for rescuing sutures fell within a narrow range. Any dramatic alteration in the amount of TGF-β3 present, either by removing the dura mater as a source, by adding neutralizing antibodies or by adding TGF-β3, resulted in too little or too much TGF-β3 in the suture region, with failure of suture maintenance. These findings are supported by the biphasic nature of TGF-β3 activity noted when the growth factor was added to osteoblasts (ten Dijke et al., 1990), with low and high concentrations of TGF-β3 resulting in reduced osteoblast cell proliferation (ten Dijke et al., 1990). Furthermore, at high concentrations, TGF-β3 induced cellular differentiation and collagen production.

The biphasic mitogenic effect of TGF-β3 on osteoblasts (ten Dijke et al., 1990) was reflected in the biphasic responsiveness of suture patency to the presence of increasing concentrations of TGF-β3. Therefore, it was decided to test whether altered rates of cell proliferation were associated with suture closure or suture rescue by examining thymidine uptake in sutures in response to addition of TGF-β2 and TGF-β3, respec-

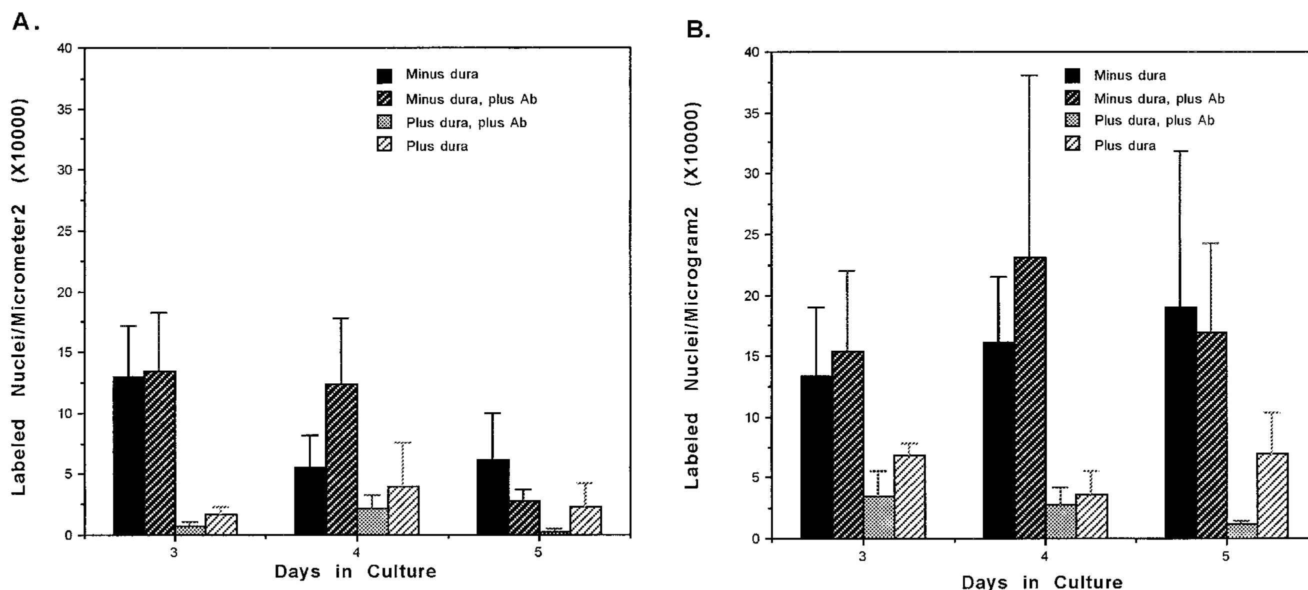

Fig. 5. Tritiated thymidine uptake in coronal sutures (**A**) and frontal and parietal bones (**B**) of F19 rat calvaria cultured in the presence (plus Ab) and absence of neutralizing antibodies to transforming growth factor-β2 established by autoradiography. No significant differences were noted between sutures or bones cultured in the presence of Ab and those cultured without Ab, after 3, 4, or 5 days in culture.

tively, to cultured fetal rat calvaria. Increased concentrations of TGF-β3 resulted in decreased levels of cell proliferation in both coronal sutures and frontal and parietal bones. In converse experiments, where sutural obliteration was induced by removal of TGF-β3 activity by neutralizing antibodies, it was found that sutural obliteration was accompanied by elevated levels of cell proliferation. This finding is similar to that seen upon removal of dura mater (Opperman et al., 1998); however, in those experiments, fusion of sutures was noted later than that seen here. Interestingly, at later culture times, the dose dependence of cell proliferation on amount of TGF-β3 present disappears and all sutures receiving TGF-β3 show decreased cell proliferation. Once the process of suture fusion is either firmly instituted or completed as in control sutures cultured in the absence of dura mater, cell proliferation returns to background levels, seen by 5 days in culture. These data provided good evidence for abnormally elevated cell numbers being a contributory factor to premature obliteration of cranial sutures, perhaps by producing a critical density at which cells are induced to differentiate (Frenkel et al., 1990, 1992; Mayahara et al., 1993). Furthermore, the biphasic effect of TGF-β3 on suture patency was reflected in the rates of DNA synthesis. Increasing concentrations of TGF-β3 initially led to decreased amounts of DNA synthesis associated with lack of complete fusion of the sutures, but elevated levels of DNA synthesis accompanied further increases in TGF-β3. This was the reverse of that noted in osteoblast cell culture (ten Dijke et al., 1990). These differences may well reflect significant differences in environment and cell number in tissues compared with isolated cell culture. Interestingly, although elevated levels of cell proliferation accompanied suture fusion induced by addition of TGF-β2, rescue of sutures from obliteration by addition of TGF-β2 neutralizing antibodies was not accompanied by a decline in cell proliferation. This leads to the speculation that TGF-β2 regulates both cell proliferation and differentiation in this tissue and that the neutralizing antibodies removed the differentiative function of TGF-β2, allowing the sutures to remain open even in the presence of increased cell density. Support for the notion that cell proliferation is associated with premature suture closure is provided by evidence that addition of FGF-4 to cultured fetal mouse calvaria induced premature suture fusion, associated with elevated levels of cell proliferation (Kim et al., 1998), as did overexpression of MSX2 (Liu et al., 1999).

In contrast to these findings, the recent results of Iseki et al. (1999) indicated that FGF-2- induced suture closure was associated with decreased levels of cell proliferation, a change from FGFR2 to FGFR1 expression and increased expression of osteopontin, a marker for osteoblast differentiation. These data were supported by the work of De Pollak et al. (1996) and Fragale et al. (1999), who demonstrated that osteoblasts from craniosynostotic patients showed low levels of cell proliferation, accompanied by elevated markers for differentiation. Experiments by Lomri et al. (1998) demonstrated that osteoblasts with FGFR2 mutations resulting in the Apert phenotype appeared to have normal proliferative responses to FGF-2, but with accelerated differentiation and bone formation. Interestingly, normal human osteoblasts increased TGF-β2

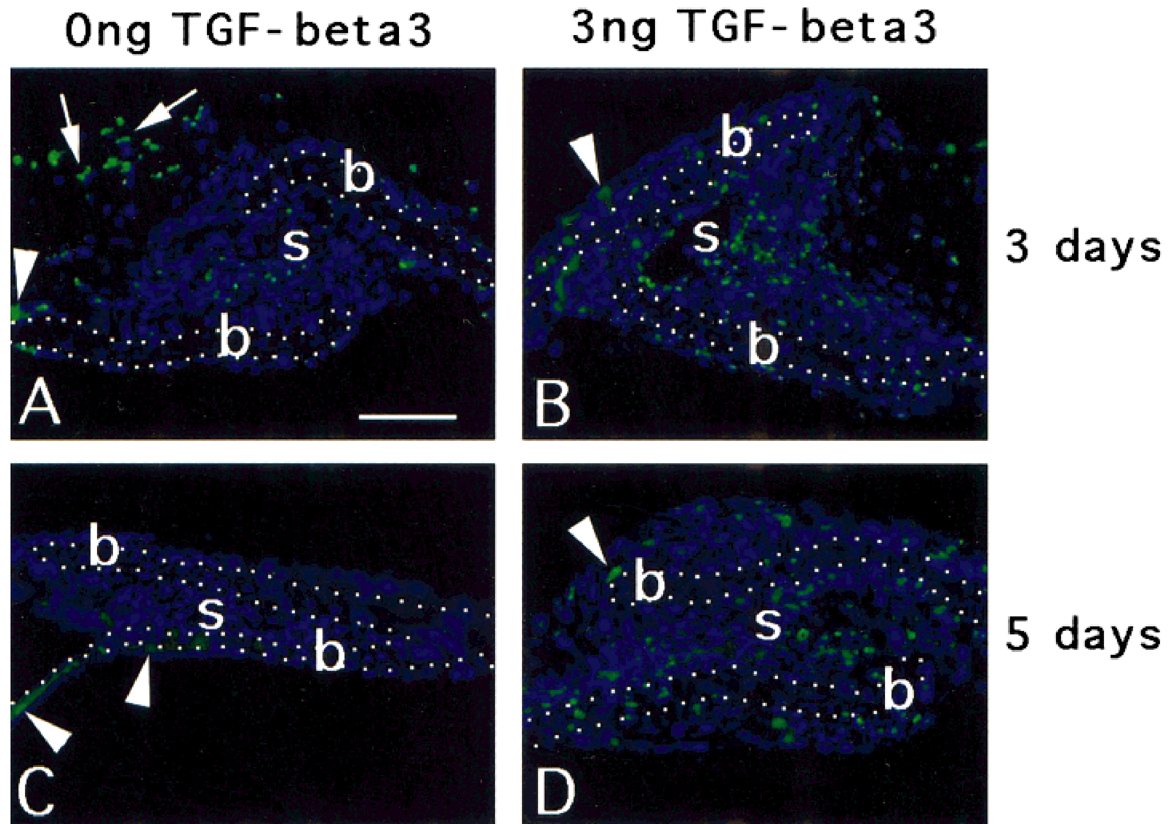

Fig. 6. Photomicrographs of F19 rat calvaria cultured without dura mater for 3 and 5 days in the presence or absence of 3 ng/ml transforming growth factor (TGF) -β3, showing immunofluorescent staining for apoptotic cell nuclei (fluorescein, green) with a counterstain for all nuclei (4,6-diamidino-2-phenylindole, blue). Note the low level of staining for apoptotic nuclei in the suture region (s), between overlapping bone fronts (b, dashed lines) of calvaria cultured without TGF-β3 (**A,C**). Bone fronts are fused across suture after 5 days in culture (C). Elevated numbers of apoptotic nuclei are present in suture region of calvaria cultured in the presence of TGF-β3 (**B,D**). Suture is still intact after 5 days in culture (D). Apoptotic nuclei are also present in the periosteum (arrows) and osteoblasts lining the bone fronts (arrowheads). Scale bar = 150 μm in A (applies to A–D).

production during prolonged exposure to FGF-2, accompanied by increased osteocalcin production and matrix mineralization (Debiais et al., 1998). Results from present studies demonstrated that rescue of sutures from obliteration was achieved by removal of TGF-β2 activity without altering cell proliferation, in contrast to the initial hypothesis. Taken together, these results suggest that mechanisms other than adjustment of levels of cell proliferation are involved in regulating cell number and suture patency.

Another mechanism by which abnormal numbers of cells could contribute to the premature obliteration of sutures would be by altering the number of apoptotic cells in the suture. Rice et al. (1999) have shown that apoptosis occurs during normal suture morphogenesis in the cells lining the bone fronts and particularly at the leading edges of the overlapping bones within the suture. They proposed that apoptosis was a part of normal suture development and suggested that increased apoptosis could be associated with delayed suture closure as in cleidocranial dysplasia, whereas decreased apoptosis could result in premature suture fusion. Because both FGF-2 and FGF-4 inhibited apoptosis in the interdigital areas of chick limb buds (Macias et al., 1996) leading to webbed digits and both factors induced suture obliteration (Iseki et al., 1997; Kim et al., 1998), it is likely apoptosis plays a role in regulating suture patency.

Results reported here demonstrated that increased numbers of apoptotic cells accompanied decreased cell proliferation both during normal suture maintenance and during rescue of sutures from obliteration with TGF-β3. Furthermore, low numbers of apoptotic cells accompanied elevated levels of cell proliferation in sutures induced to fuse by removal of dura mater or by addition of TGF-β2. This supports the mechanism predicted by Rice et al. (1999). An alternate role for apoptosis was first presented by Furtwangler et al. (1985), who suggested that apoptotic cells would be found at the edges of bone fronts, which become too closely approximated, preventing sutural obliteration. This mechanism is supported by the finding here that elevated levels of apoptotic cells were found along the bone fronts of sutures not undergoing fusion. However,

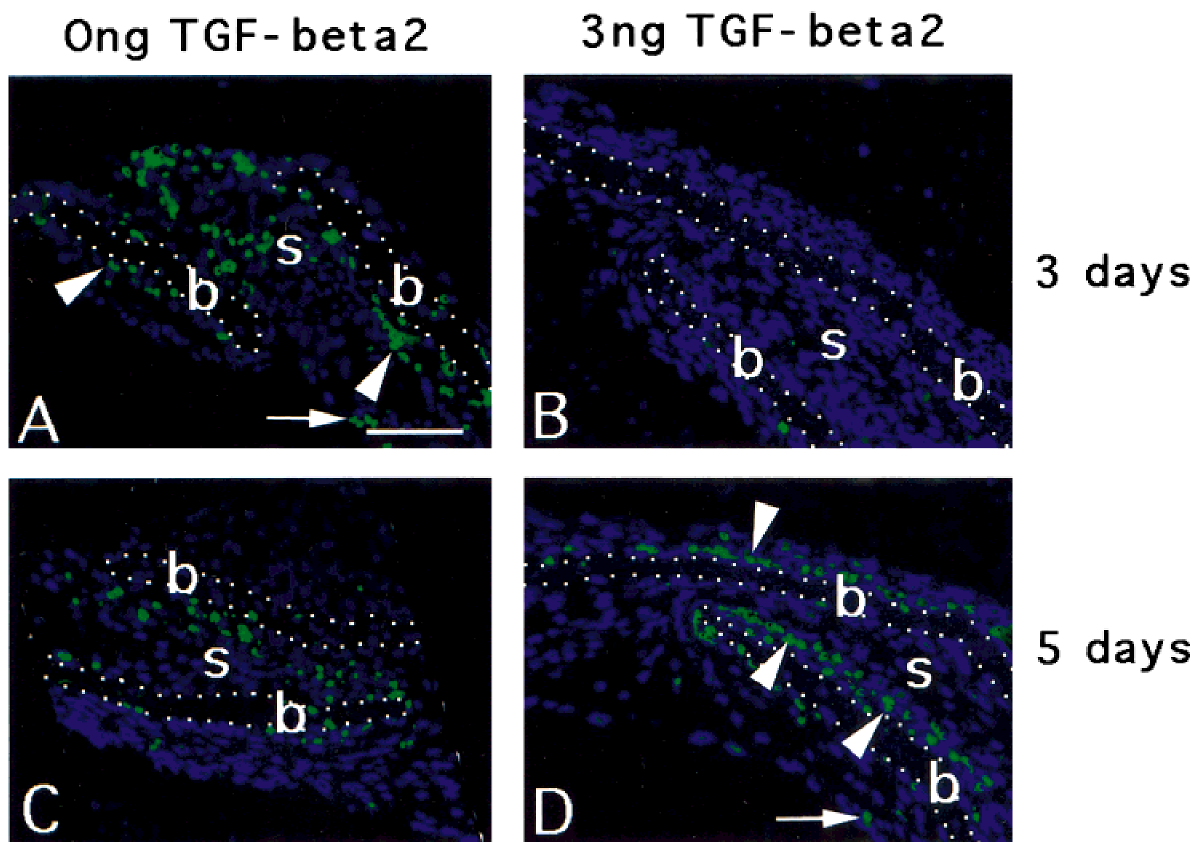

Fig. 7. Photomicrographs of F19 rat calvaria cultured with intact dura mater for 3 and 5 days in the presence or absence of 3 ng/ml transforming growth factor (TGF) -β2, showing immunofluorescent staining for apoptotic cell nuclei (fluorescein, green) with a counterstain for all nuclei (4,6-diamidino-2-phenylindole, blue). **A,C:** Note high numbers of apoptotic cells in suture region (s) between overlapping bone fronts (b, dashed lines) in calvaria cultured without TGF-β2. **B,D:** Addition of TGF-β2 results in decreased numbers of apoptotic nuclei within the suture. Apoptotic cells are visible lining the bone fronts of calvaria (arrowheads) and within the dura (arrows). Scale bar = 125 μm in A (applies to A–D).

this was not sufficient to prevent obliteration, because apoptotic cells were also found along the bone fronts of sutures induced to fuse by addition of TGF-β2. The evidence suggests that for sutures to remain intact, a critical number of cells within the sutural matrix must become apoptotic. Without apoptosis, the number of cells within the suture may surpass a critical density, triggering differentiation and resulting in bone formation and suture obliteration. This is supported by the finding that increased cell density is associated with differentiation (Frenkel et al., 1990, 1992; Mayahara et al., 1993). However, it is possible that cells could be triggered to differentiate prematurely without initially increased cell numbers, by premature differentiation of normally nondifferentiating cells within the suture.

By using the same chick limb model system described above (Macias et al., 1996) as an example, an additional mechanism can be proposed. TGF-β2 added to the interdigital areas of chick limb buds resulted in inhibition of apoptosis and formation of ectopic cartilage (Ganan et al., 1996). This chondrogenic effect was antagonized by addition of FGF-2. Addition of either TGF-β2 or FGF-2 resulted in inhibition of apoptosis; however, the former led to cell differentiation, whereas the latter led to increased cell proliferation. In a similar manner in the suture then, the combined actions of these growth factors could increase cell density and initiate premature cell differentiation, resulting in suture obliteration. TGF-β3 could act as an antagonist to these actions, serving to maintain sutures in their patent state.

These findings, summarized in Figure 8, led to the formulation of the following model. In human pathology, the activating mutations in some FGF receptors (Deng et al., 1996; Galvin et al., 1996; Anderson et al., 1998; Naski et al., 1998) simulate addition of FGF-2 and FGF-4 by inhibiting apoptosis in sutural and perisutural tissues. Elevated levels of cell proliferation may accompany this event. The resulting increased numbers of cells would change predominant FGF receptor expression from FGFR2 to FGFR1 and cells would begin to differentiate under the influence of elevated levels of TGF-β2, resulting in bony obliteration of sutures. The prediction that elevated levels of TGF-β2 would be found in synostotic sutures has some evidence in the literature (Lin et al., 1997). On the

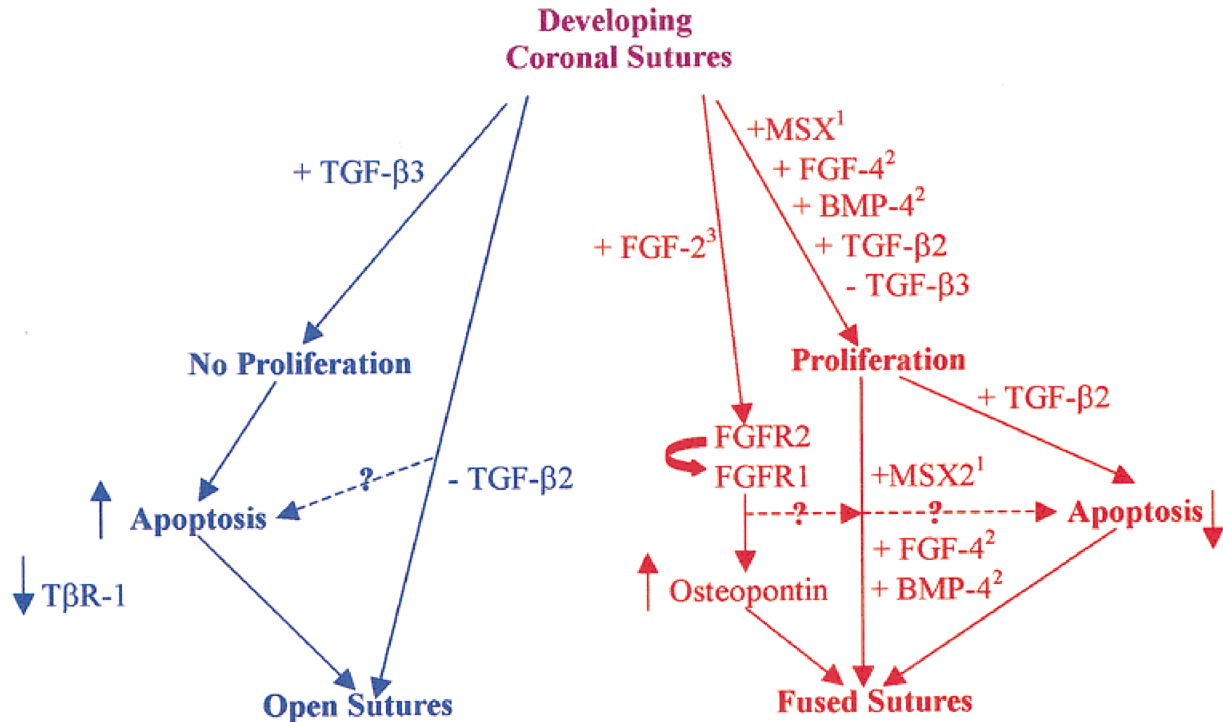

Fig. 8. Schematic showing the relationship between addition or removal of growth factors and cellular events associated with regulating cell number and cell differentiation. Increased proliferation, decreased apoptosis and changes in fibroblast growth factor receptors expression patterns are associated with events leading to suture fusion. Lack of proliferation, increased apoptosis and down-regulation of TβR-1 are associated with maintenance of sutures in their patent state. [1], Liu et al., 1999; [2], Kim et al., 1998; [3], Iseki et al., 1997.

other hand TGF-β3, by regulating cell number through altered rates of cell proliferation and apoptosis would provide protection of sutures from premature obliteration. Although it would be convenient to speculate that TGF-β3 could also regulate TGF-β2 levels, either directly or by means of regulation of FGF or FGFR expression, immunohistochemical data presented here do not support this. However, unpublished preliminary data (Opperman) indicate TGF-β3 may down-regulate TGF-β receptor (TβR)-1 expression, which would attenuate TGF-β2 signaling. Studies currently are under way to examine levels of apoptosis in sutures in response to stimulation by FGF and to test whether TGF-β3 exerts its protective effects further by regulating FGFR and TβR-1 and TβR-2 expression.

EXPERIMENTAL PROCEDURES

Preparation and Culture of Fetal Calvaria

Calvarial rudiments from 19-day-old (F19) Sprague-Dawley (Harlan) rat fetuses (date of mating plug = day 0) were prepared as previously described (Opperman et al., 1995). Calvaria either with intact dura mater or with dura mater removed were cultured as follows: Group I, no TGF-β or neutralizing antibodies; group II, either 0.03, 0.3, 3.0, or 30 ng/ml TGF-β3; group III, 3 ng/ml TGF-β2; group IV, either neutralizing antibodies to TGF-β3 or to TGF-β2 as previously detailed (Opperman et al., 1999). TGF-β2 and TGF-β3 antigens as well as their polyclonal neutralizing antibodies were obtained from R & D Systems. Calvaria were placed in 24-well plates (Costar), with the dura mater side down, and covered with 400 μl of medium (Opperman et al., 1995), to which 100 μg/ml ascorbic acid was added daily. TGF-βs or neutralizing antibodies were added at feeding time every 2 days. Calvaria from 10 experiments were pooled for analysis of suture patency, rates of DNA synthesis, and levels of apoptosis.

Suture Histomorphometry and Statistical Analysis

Tissues were harvested after 3, 4, and 5 days in culture, and prepared for histology as described previously (Opperman et al., 1995). Sections were randomly assigned and scored by two observers blinded to experimental procedure. Briefly, each section was scored for degree of osseous obliteration of sutures (open sutures = O; bone fronts touching across narrowed suture but not fused = N; bone fronts fused in region of suture = F). The final numbers of explants for each experimental condition are given in Table 1.

Calculation of DNA Synthesis in the Presence of TGF-β2 and TGF-β3

To assess rates of DNA synthesis, cultures were pulse-labeled with 10 μl of [methyl-^{3}H]thymidine (1 mCi/ml) (NEN, specific activity 8 TBq/mmol) added to the culture medium 18 hr before harvest. Tissues were harvested after 3, 4, and 5 days in culture. All tissues were washed six times in calcium and magnesium free phosphate-buffered saline (PBS; 2.7 mM KCl, 140 mM NaCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) to remove unincorporated counts. Tissues were then washed three times in acetone and air-dried overnight. The calvaria were microdissected into pieces comprising coronal sutures, frontal bones, parietal bones (n = 2 for each calvaria), and intrafrontal and sagittal sutures. Each piece of tissue was weighed by using a C-30 microbalance (Cahn) before being placed in a 7-ml Ready-Safe scintillation cocktail (Beckman) and counted in a Tricarb 2900TR Beta Counter (Packard). All counts were presented as disintegrations per minute per microgram of dry weight of tissue for statistical analysis. Either analysis of variance with Tukey-Kramer multiple comparisons test (TGF-β2) or regression analysis (TGF-β3) was used. Tissues from multiple runs were pooled to produce enough calvaria and each time point, and experimental condition was repeated at least three times.

Calculation of DNA Synthesis in the Presence of Neutralizing Antibodies to TGF-β2 and TGF-β3

Tissues cultured with neutralizing antibodies were pulse-labeled for 18 hr with tritiated thymidine, harvested and washed in PBS as described above, before being prepared for histology and autoradiography as described previously (Opperman et al., 1998). Numbers of labeled nuclei/μm^2 were calculated after image capture by CCD camera (Pixera) and differences statistically tested by analysis of variance (Opperman et al., 1998). Data are presented as means ± SD.

Immunohistochemical Analysis of TGF-β2 Distribution in Response to TGF-β3 Administration

An indirect immunoperoxidase procedure was carried out as described previously (Opperman et al., 1997). Briefly, endogenous peroxidase activity was removed by preincubation in 4:1 methanol: 3% hydrogen peroxide. PBS containing 2% normal rabbit serum (Sigma) was used as blocker before application of primary TGF-β2 polyclonal antibodies company tested for specificity by Western blotting procedures (1:500, R & D Systems). Control slides were incubated in the absence of primary antibody. Secondary antibodies were peroxidase-conjugated rabbit anti-goat IgG (1:500, Jackson Immunoresearch Laboratories) with diaminobenzidine (Sigma) as chromagen. Immunostaining was scored as absent (-), low (+), medium (++), or high (+++).

Apoptosis Assays

Apoptag® apoptosis assay kits (Intergen), with fluorescein (FITC) were used to test for presence of apoptotic cells on histologic sections. Parasagittal sections through the coronal sutures of F19 rat calvaria cultured either with intact dura mater and treated with 0 or 3 ng/ml TGF-β2 or cultured with dura mater removed and treated with 0 or 3 ng/ml TGF-β3 were assayed. Coverslips were applied with 4:1 Flouromount-G® (Southern Biotechnology Association):Vectashield® (Vector Laboratories) containing 4,6-diamidino-2-phenylindole (DAPI) to label all nuclei blue as counterstain. Images were captured by using a Nikon microscope with Metamorph® software (Nikon). DAPI and FITC images were superimposed by using Adobe Photoshop® software.

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REFERENCES

Anderson J, Burns HD, Enriquez-Harris P, Wilkie AOM, Heath JK. 1998. Apert syndrome mutations in fibroblast growth factor receptor 2 exhibit increased affinity for FGF ligand. Hum Mol Genet 7:1475–1483.

Baer MJ. 1954. Patterns of growth of the skull as revealed by vital staining. Hum Biol 26:80–126.

Bellus GA, Gaudenz K, Zackai EH, Clarke LA, Szabo J, Francomano CA, Muenke M. 1996. Identical mutations in three different fibroblast growth factor receptor genes in autosomal dominant craniosynostosis syndromes. Nat Genet 14:174–176.

Cohen MM Jr. 1993. Sutural biology and the correlates of craniosynostosis. Am J Med Genet 47:581–616.

De Pollak C, Renier D, Hott M, Marie PJ. 1996. Increased bone formation and osteoblastic cell phenotype in premature cranial suture ossification (craniosynostosis). J Bone Miner Res 11:401–407.

Debiais F, Hott M, Graulet AM, Marie PJ. 1998. The effects of fibroblast growth factor-2 on human neonatal calvaria osteoblastic cells are differentiation stage specific. J Bone Miner Res 13:645–654.

Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P. 1996. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell 84:911–921.

el Ghouzzi V, Le Merrer M, Perrin-Schmitt F, Lajeunie E, Benit P, Renier D, Bourgeois P, Bolcato-Bellemin AL, Munnich A, Bonaventure J. 1997. Mutations of the TWIST gene in the Saethre-Chotzen syndrome. Nat Genet 15:42–46.

Enlow DH. 1989. Normal and abnormal patterns of craniofacial growth. In: Persing JA, Edgerton MT, Jane JA, editors. Scientific foundations and surgical treatment of craniosynostosis. Baltimore: Williams and Wilkins. p 83–86.

Fragale A, Tartaglia M, Bernardini S, Di Stasi AM, Di Rocco C, Velardi F, Teti A, Battaglia PA, Migliaccio S. 1999. Decreased proliferation and altered differentiation in osteoblasts from genetically and clinically distinct craniosynostotic disorders. Am J Pathol 154:1465–1477.

Frenkel SR, Grande DA, Collins M, Singh IJ. 1990. Fibroblast growth factor in chick osteogenesis. Biomaterials 11:38–40.

Frenkel SR, Herskovits MS, Singh IJ. 1992. Fibroblast growth factor: effects on osteogenesis and chondrogenesis in the chick embryo. Acta Anat 145:265–268.

Furtwangler JA, Hall SH, Koskinen-Moffett LK. 1985. Sutural morphogenesis in the mouse calvaria: the role of apoptosis. Acta Anat 124:74–80.

Galvin BD, Hart KC, Meyer AN, Webster MK, Donoghue DJ. 1996. Constitutive receptor activation by Crouzon syndrome mutations in fibroblast growth factor receptor (FGFR)2 and FGFR2/Neu chimeras. Proc Nat Acad Sci USA 93:7894–7899.

Ganan Y, Macias D, Duterque-Coquillaud M, Ros MA, Hurle JM. 1996. Role of TGF beta s and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. Development 122:2349–2357.

Howard TD, Paznekas WA, Green ED, Chiang LC, Ma N, Ortiz de Luna RI, Garcia Delgado C, Gonzalez-Ramos M, Kline AD, Jabs EW. 1997. Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. Nat Genet 15:36–41.

Iseki S, Wilkie AO, Heath JK, Ishimaru T, Eto K, Morriss-Kay GM. 1997. Fgfr2 and osteopontin domains in the developing skull vault are mutually exclusive and can be altered by locally applied FGF2. Development 124:3375–3384.

Iseki S, Wilkie AO, Morriss-Kay GM. 1999. Fgfr1 and Fgfr2 have distinct differentiation- and proliferation-related roles in the developing mouse skull vault. Development 126:5611–5620.

Jabs EW, Li X, Scott AF, Meyers G, Chen W, Eccles M, Mao JI, Charnas LR, Jackson CE, Jaye M. 1994. Jackson-Weiss and Crouzon syndromes are allelic with mutations in fibroblast growth factor receptor 2. Nat Genet 8:275–279.

Jabs EW, Muller U, Li X, Ma L, Luo W, Haworth IS, Klisak I, Sparkes R, Warman ML, Mulliken JB, et al. 1993. A mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis. Cell 75:443–450.

Kim HJ, Rice DP, Kettunen PJ, Thesleff I. 1998. FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. Development 125:1241–1251.

Lin KY, Nolen AA, Gampper TJ, Jane JA, Opperman LA, Ogle RC. 1997. Elevated levels of transforming growth factors beta 2 and beta 3 in lambdoid sutures from children with persistent plagiocephaly. Cleft Palate Craniofac J 34:331–337.

Liu YH, Tang Z, Kundu RK, Wu L, Luo W, Zhu D, Sangiorgi F, Snead ML, Maxson RE. 1999. Msx2 gene dosage influences the number of proliferative osteogenic cells in growth centers of the developing murine skull: a possible mechanism for MSX2-mediated craniosynostosis in humans. Dev Biol 205:260–274.

Lomri A, Lemonnier J, Hott M, de Parseval N, Lajeunie E, Munnich A, Renier D, Marie PJ. 1998. Increased calvaria cell differentiation and bone matrix formation induced by fibroblast growth factor receptor 2 mutations in Apert syndrome. J Clin Invest 101:1310–1317.

Ma L, Golden S, Wu L, Maxson R. 1996. The molecular basis of Boston-type craniosynostosis: the Pro148→His mutation in the N-terminal arm of the MSX2 homeodomain stabilizes DNA binding without altering nucleotide sequence preferences. Hum Mol Genet 5:1915–1920.

Macias D, Ganan Y, Ros MA, Hurle JM. 1996. In vivo inhibition of programmed cell death by local administration of FGF-2 and FGF-4 in the interdigital areas of the embryonic chick leg bud. Anat Embryol (Berl) 193:533–541.

Mayahara H, Ito T, Nagai H, Miyajima H, Tsukuda R, Taketomi S, Mizoguchi J, Kato K. 1993. In vivo stimulation of endosteal bone formation by basic fibroblast growth factor in rats. Growth Factors 9:73–80.

Meyers GA, Day D, Goldberg R, Daentl DL, Przylepa KA, Abrams LJ, Graham JM Jr, Feingold M, Moeschler JB, Rawnsley E, Scott AF, et al. 1996. FGFR2 exon IIIa and IIIc mutations in Crouzon, Jackson-Weiss, and Pfeiffer syndromes: evidence for missense changes, insertions, and a deletion due to alternative RNA splicing. Am J Hum Genet 58:491–498.

Muenke M, Gripp KW, McDonald-McGinn DM, Gaudenz K, Whitaker LA, Bartlett SP, Markowitz RI, Robin NH, Nwokoro N, Mulvihill JJ, Losken HW, et al. 1997. A unique point mutation in the fibroblast growth factor receptor 3 gene (FGFR3) defines a new craniosynostosis syndrome. Am J Hum Genet 60:555–564.

Muenke M, Schell U, Hehr A, Robin NH, Losken HW, Schinzel A, Pulleyn LJ, Rutland P, Reardon W, Malcolm S, et al. 1994. A common mutation in the fibroblast growth factor receptor 1 gene in Pfeiffer syndrome. Nat Genet 8:269–274.

Naski MC, Colvin JS, Coffin JD, Ornitz DM. 1998. Repression of hedgehog signaling and BMP4 expression in growth plate cartilage by fibroblast growth factor receptor 3. Development 125:4977–4988.

Opperman LA, Chhabra A, Cho RW, Ogle RC. 1999. Cranial suture obliteration is induced by removal of transforming growth factor (TGF)-beta 3 activity and prevented by removal of TGF-beta 2 activity from fetal rat calvaria in vitro. J Craniofac Genet Dev Biol 19:164–173.

Opperman LA, Chhabra A, Nolen AA, Bao Y, Ogle RC. 1998. Dura mater maintains rat cranial sutures in vitro by regulating suture cell proliferation and collagen production. J Craniofac Genet Dev Biol 18:150–158.

Opperman LA, Nolen AA, Ogle RC. 1997. TGF-beta 1, TGF-beta 2, and TGF-beta 3 exhibit distinct patterns of expression during cranial suture formation and obliteration in vivo and in vitro. J Bone Miner Res 12:301–310.

Opperman LA, Passarelli RW, Morgan EP, Reintjes M, Ogle RC. 1995. Cranial sutures require tissue interactions with dura mater to resist osseous obliteration in vitro. J Bone Miner Res 10: 1978–1987.

Opperman LA, Passarelli RW, Nolen AA, Gampper TJ, Lin KY, Ogle RC. 1996. Dura mater secretes soluble heparin-binding factors required for cranial suture morphogenesis. In Vitro Cell Dev Biol 32:627–632.

Opperman LA, Sweeney TM, Redmon J, Persing JA, Ogle RC. 1993. Tissue interactions with underlying dura mater inhibit osseous obliteration of developing cranial sutures. Dev Dyn 198:312–322.

Reardon W, Winter RM, Rutland P, Pulleyn LJ, Jones BM, Malcolm S. 1994. Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome. Nat Genet 8:98–103.

Rice DP, Kim HJ, Thesleff I. 1997. Detection of gelatinase B expression reveals osteoclastic bone resorption as a feature of early calvarial bone development. Bone 21:479–486.

Rice DP, Kim HJ, Thesleff I. 1999. Apoptosis in murine calvarial bone and suture development. Eur J Oral Sci 107:265–275.

Roth DA, Gold LI, Han VK, McCarthy JG, Sung JJ, Wisoff JH, Longaker MT. 1997a. Immunolocalization of transforming growth factor beta 1, beta 2, and beta 3 and insulin-like growth factor I in premature cranial suture fusion. Plast Reconstr Surg 99:300–309.

Roth DA, Longaker MT, McCarthy JG, Rosen DM, McMullen HF, Levine JP, Sung J, Gold LI. 1997b. Studies in cranial suture biology: I. Increased immunoreactivity for TGF-beta isoforms (beta 1, beta 2, and beta 3) during rat cranial suture fusion. J Bone Miner Res 12:311–321.

ten Dijke P, Iwata KK, Goddard C, Pieler C, Canalis E, McCarthy TL, Centrella M. 1990. Recombinant transforming growth factor type beta 3: biological activities and receptor-binding properties in isolated bone cells. Mol Cell Biol 10:4473–4479.

Wilkie AO, Slaney SF, Oldridge M, Poole MD, Ashworth GJ, Hockley AD, Hayward RD, David DJ, Pulleyn LJ, Rutland P, et al. 1995. Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. Nat Genet 9:165–172.