Platelet-derived growth factor (PDGF) is known to stimulate osteoblast or osteoprogenitor cell activity. We investigated the effect of locally applied PDGF from poly-D,L-lactide (PDLLA)-coated implants on fracture healing in a rat model. A closed fracture of the right tibia of four-month-old Sprague-Dawley rats (n = 40) was stabilised with implants coated with a biodegradable PDLLA versus implants coated with PDLLA and PDGF. Radiographs were taken throughout the study, and a marker of DNA activity, bromodeoxyuridine (BrdU), was injected before the rats were killed at three, seven and ten days. The radiographs showed consolidation of the callus in the PDGF-treated group compared with the control group at all three time points. In the PDGF-treated group, immunohistochemical staining of BrdU showed that the distribution of proliferating cells in all cellular events was higher after ten days compared with that at three and seven days.

These results indicate that local application of PDGF from biodegradable PDLLA-coated implants significantly accelerates fracture healing in experimental animals. Further development may help fracture healing in the clinical situation.

Fracture repair begins with bleeding at the fracture site followed by an inflammatory reaction, chondrogenesis, intramembranous and endochondral ossification, and various types of cellular proliferation,1,2 influenced by growth factors.3,4 It is known that platelet-derived growth factor (PDGF) increases bone matrix apposition in a dose-dependent manner and is capable of stimulating bone formation.5-7 The local and controlled application of this factor could be effective in stimulating fracture healing.8 A stimulating effect on fracture healing due to the locally applied combination of other growth factors, such as insulin growth factor-1 (IGF-1) and transforming growth factor-β1 (TGF-β1) on poly-(D,L-lactide) (PDLLA)-implants was found in a rat study investigating their osseo-inductive effects five, ten and 15 days after fracture.9 Moreover, a dose-dependent effect of accelerated healing in bone defects was observed using bone morphogenetic protein-2 (BMP-2) on a porous PDLLA carrier in a rabbit model.10 Incorrect placement of some application devices might induce heterotopic bone formation in the surrounding soft tissues, such as muscles, nerves and blood vessels. In order to reduce these risks and the potential side effects, coating of implants could produce high local concentrations of incorporated growth factors. Because of the very short half-life of growth factors (60 mins to 240 mins),11 a recently developed cold coating technique for local protein delivery could potentially fulfil these requirements.12 This coating is based on biodegradable PDLLA as carrier and allows the incorporation of growth factors and their controlled release from the implant surface during the healing process. This implant could act both as a device for fracture stabilisation and as a method for delivering chemicals to stimulate fracture healing. Cell proliferation at the fracture site can be detected by the injection of the thymidine analogue bromodeoxyuridine (BrdU) before death.13 The incorporation of BrdU into DNA has been very useful in different biological systems to determine the fraction of the S phase cells,13 and can be used to identify by immunohistochemistry cells proliferating at the fracture site, which may include osteoprogenitor cells.

The purpose of this study was to investigate in a rat model the effect of PDGF released locally from coated implants on fracture healing, which may have a clinically useful application in the future.

Materials and Methods
Coating techniques and application of growth factor. PDLLA (1 mg, 30 kDa) (Sigma Aldrich Co., St Louis, Missouri), was dissolved in chloroform and PDGF (both Sigma Aldrich) was incorporated. Titanium Kirschner (K-) wires 1.0 mm in diameter (Sigma Aldrich)
were twice coated over their entire length and dried under sterile conditions. The incorporated dose of growth factor in the coating was 50 μg PDGF (each implant 5%, w/w). The properties of the coating and the release characteristics of the incorporated growth factor have been described previously. The following groups were examined: group 1, control, implants coated with PDLLA (n = 20), and group 2, implants coated with PDLLA and PDGF (n = 20).

**Animal and fracture model.** Right tibiae from 40 four-month-old male Sprague-Dawley rats (mean weight 250 g (SD 6)) (Charles River, Wilmington, Massachusetts) were fractured using a fracture device. After sedation with Vetocaine (Agrovet Market, Lima, Peru) and intraperitoneal anaesthesia using a mixture of ketamine hydrochloride (100 mg/ml at 60 mg/kg body weight) and 2% xylazine (10 mg/kg body weight) (both Sigma Aldrich), the right leg was shaved and disinfected. A 3 mm incision was performed at the tuberositas tibiae and the cortical bone and medullary canal were opened using a 1 mm steel K-wire. After removing the wire, the right tibia was fractured with a fracture device described elsewhere. It was externally reduced of the fracture the tibiae were stabilised with the K-wire implants coated with either PDLLA or PDLLA and PDGF. They were inserted from the proximal end of the tibia into the medullary canal. After stabilisation, radiographs were taken to document the fracture. The rats were chosen for sacrifice randomly in a blinded fashion at three, seven and ten days, and prepared for immunohistochemistry. All animals used in this study were maintained in a facility accredited by the Helsinki Declaration and according to the guidelines of the Ethics Committee of the International Association for the Study of Pain EC Directive 86/609/EEC. Also, animals received humane care in accordance with the National Institutes of Health’s Guide for Care and Use of Laboratory Animals.

**Radiological evaluation.** Posteroanterior and lateral radiographs were taken in eight animals chosen randomly from each group at three, seven and ten days. Digital radiographs were taken using a Mobiunit Plus X-ray unit (Siemens AG, Munich, Germany) and the bridging (mineralisation) of the fracture callus was described by two independent observers using the following parameters: A) complete bridging (four cortices bridged); B) incomplete bridging (one to three cortices bridged); and C) no bridging (no cortex bridged).

**BrdU injection.** The BrdU was dissolved 1:4 in dimethyl sulfoxide (Sigma Aldrich) to extend its release and administered subcutaneously five hours before sacrifice at a rate of 30 mg/kg body weight to five animals from each group. In order to determine the optimum time of five hours for the BrdU injection, longer timepoints were chosen and analysed in a pilot study (five, ten and 15 hours before death, one rat at each time point).

**Immunohistochemical staining.** After death the tibiae were dissected out, fixed for two days in 10% normal buffered formaldehyde, decalcified in ethylenediaminetetra-acetic acid, and embedded in paraffin wax before conventional 4 μm histological sections were cut. These were mounted in poly-L-lysine-coated glass slides and then air dried overnight at room temperature. The paraffin was removed with xylene and hydrated with serial concentrations of 100%, 95%, 80% and 70% alcohol. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 60 minutes. The slides were washed in tris-buffered saline (TBS)/0.1% bovine serum albumin (BSA), and hyaluronidase treatment (hyaluronidase 1 mg/ml in sodium acetate buffer, pH 5.5), 0.85% sodium chloride was performed for 30 minutes at 37°C. The sections were then washed with TBS/0.1% BSA, and incubated with a blocking solution of 10 ml fresh TBS/0.5% BSA and 130 μl of normal horse serum at room temperature for 15 minutes, followed by incubation with monoclonal antibody BrdU (1:200 dilution). After this a reaction was made using a Vectastain avidin–biotin–peroxidase complex kit followed by a 3,3′-diaminobenzidine (DAB) solution and counterstaining with methyl green. The morphology and the proliferating cells in the callus were analysed with an image analysis system (KS400; Carl-Zeiss, Microimaging GmbH, Standort Göttingen, Germany). Purified mouse IgG (all reagents from Sigma Aldrich) was used as a control primary antibody.

**Quantification of proliferating cells.** The intensity and extent of positive staining by BrdU labelling were measured using an image analysis computer system (Nexus Qube image analyser processor; Nexus Inc., Tokyo, Japan), and the animals were again randomly chosen in a blinded fashion for the analysis. This video-based system distinguished the density of brown-DAB reaction product from the nuclei stained with methyl green and the ratio of this area of brown-DAB reaction to the total area of cell nuclei was calculated as the BrdU score. The measurements were carried out at least three times at one location for each specimen, and the mean value obtained was used as the BrdU score. The tibia was treated with the same procedures, and the immunohistochemistry was carried out on the same glass slides. In order to negate any differences in the immunostaining conditions between each specimen at different times after the insertion of the K-wires, the score of purified mouse IgG was used as a control primary antibody.

**Statistical analysis.** Data were compared using one-way analysis of variance (ANOVA) for independent samples. The radiological score was analysed using the chi-squared test. Both tests were controlled using Bonferroni’s correction. Interobserver variability for the radiological evaluation was determined using κ statistics.
differences were defined at the 95% confidence level. Values are expressed as mean and standard deviation (SD).

Results

Radiological examinations. Interobserver agreement for radiological evaluation was showing a κ value of 0.91. The coated implant group showed enhanced consolidation of the fractures after ten days compared with controls (Fig. 1). In group 1 the gap was not bridged completely after three days in three of eight animals, after seven days in five of eight animals and after ten days in four of eight animals. The tibiae in group 2 were bridged completely in five of eight animals in three days, in six of eight animals after seven days, and in seven of eight animals after ten days. There was a significantly higher rate of fracture consolidation at ten days (p < 0.05) in group 2 than in group 1 (Table I). The animals found to have no radiologically detectable bridging callus showed no mineralised callus bridging but rather a soft callus, which was on subsequent immunostaining analyses. Interestingly, no heterotopic bone formation was detected at the proximal insertion area in any group.

Immunostaining analysis. The BrdU injected before sacrifice is incorporated into DNA and highlights the osteoblasts, osteoprogenitor cells, or cells that proliferated during the incubation time of the BrdU in vivo. As shown in Figure 2, the number of labelled cells depends on the injection and incubation time of the BrdU. No bridging, Incomplete bridging, Complete bridging.

Table I. Radiological score (n = 8 each group) (PDGF, platelet-derived growth factor)

<table>
<thead>
<tr>
<th></th>
<th>3 days Uncoated</th>
<th>7 days Uncoated</th>
<th>10 days Uncoated</th>
<th>3 days PDGF-coated</th>
<th>7 days PDGF-coated</th>
<th>10 days PDGF-coated</th>
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<tr>
<td>No bridging</td>
<td>3</td>
<td>0 †</td>
<td>5</td>
<td>2 †</td>
<td>4</td>
<td>1 †</td>
</tr>
<tr>
<td>Incomplete bridging</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Complete bridging</td>
<td>3</td>
<td>5 †</td>
<td>1</td>
<td>6 †</td>
<td>3</td>
<td>7 †</td>
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* p = 0.02 (chi-squared test) vs group 1, control † p = 0.04 (chi-squared test) vs group 1, control ‡ p < 0.05 (chi-squared test) vs group 1, control
(Figs 3c and 3d). High BrdU staining was detected in the cartilage of group 2 compared with controls. The premature cells located in the margin of the cartilage showed more staining than the hypertrophic or mature chondrocytes (Fig. 3d). After ten days, the cells in the newly formed woven bone in group 2 showed the most immunoreactivity; the proliferating cells (Figs 3e and 3f) did not show the morphology of osteoblasts but of haematopoietic cells. In the soft tissue in
group 2, more proliferating cells were detectable than in controls. No significant differences in terms of proliferating cells were found between the animals.

Quantification of BrdU-positive cells. At the inflammatory reaction site the BrdU score was about 40% in the early phase (three days), decreasing later (Fig. 4). The BrdU score during fracture repair was highest in the periosteal cells near the fracture site as early as three days after the fracture stabilisation, and decreased to one-third by day ten (Fig. 5). At the intramembranous ossification site away from the fracture site, the BrdU score was almost the same as near the fracture site at three days (20%), although it had decreased to a greater extent by day ten (5%). At the chondrogenesis site the BrdU score could not be measured using our system because the consolidation of the callus was not visible. The degree of cell proliferation from all cellular events initially increased by three days, later decreasing up to ten days. There were no significant differences between group 2 and controls.

Discussion
The aim of this study was to investigate the effect on fracture healing in an experimental model of PDGF released locally from PDLLA-coated implants. In order to assess the progress of healing, radiological analysis was carried out which showed enhanced consolidation of the fractures after ten days in group treated with coated implants compared with a control group, in which the gap was not bridged completely.

In our study only cells that incorporated the injected thymidine analogue BrdU into DNA to determine the fractions of the S phase cells were stained. However, the number of BrdU-positive cells depends on the time between injection and death. This should always be considered when an in vivo labelling method is chosen or compared with other methods. The results show that the injection of BrdU five hours before sacrifice leads to a large number of proliferating cells in the treated group. With this injection time there were fewer immunoreactive cells, but this result is in accordance with other studies investigating the same marker of proliferation at six hours before sacrifice.9
Comparison of results at different time points showed that accelerated healing could be detected in group 2 compared with group 1. After three days the callus in group 2 showed early chondrogenesis, with proliferating chondrocytes; seven days after the fracture, the cells located in the margin of the cartilage showed more staining than the hypertrophic chondrocytes; and ten days after the fracture more proliferating cells were detectable than in the controls. These results are in accordance with other studies investigating the osseo-inductive effect of BMP-2 on bone defect or fracture healing.\textsuperscript{14,18}

Our study clearly demonstrates the beneficial effect of PDGF on fracture healing. The application of growth factor did not lead to a pathological alteration of fracture healing; the changes represented an accelerated healing process.

Iwaki et al\textsuperscript{2} concluded from the proliferation patterns at different time points that this may correlate with the contemporary activity of growth factors. Although the distribution of proliferating cells and the degree of cell proliferation varied during fracture repair, current studies have shown that growth factors are involved in its local regulation. Ten hours after a fracture the distribution of proliferating cells in the callus is wide and homogeneous, most likely because of the production during haematoma formation of mitogenic growth factors, including PDGF, which disperse throughout the whole callus.\textsuperscript{19} Some studies have described the proliferation pattern of PDGF B into osteoblasts \textit{in vitro} and \textit{in vivo}.\textsuperscript{2} With respect to its local effect on bone formation, \textit{in vitro} studies have shown that PDGF inhibits proliferation by osteoblasts. It inhibits mineralisation and nodule formation and suppresses the expression of genes in long-term mineralised cultures of fetal rat calvaria.\textsuperscript{21} In contrast, \textit{in vivo} studies have shown that PDGF increases bone formation. Injection of PDGF into the medullary cavity accelerates fracture healing in tibial osteotomies in rabbits,\textsuperscript{22} and the systemic administration of PDGF increases bone density and strength in ovariectomised rats.\textsuperscript{23} In addition, PDGF mediates proliferation in almost all kinds of mesenchymal cell; therefore, it may have a complex effect, including not only direct regulation of but also indirect influences on growth factors via cytokine production induced by PDGF from other types of cells. Other growth factors, such as IGF-1 and TGF-\textit{\beta}1, have demonstrated an osseo-inductive effect on fracture healing created locally by the same PDLLA-coated implants.\textsuperscript{9,12} rather than PDGF. The current literature reflects many methods for local application of other growth factors, including collagen sponges,\textsuperscript{24} biodegradable particles, collagen gel,\textsuperscript{25} and \textit{in vivo} or \textit{ex vivo} gene therapy.\textsuperscript{26} In addition, these carriers must be placed in the region of the fracture, which requires opening the fracture site. Further relevant aspects for the choice of the method of application include the ease of manufacture, cost-effectiveness, biocompatibility and user-friendliness. Thus, much research has been performed and remains to be done to find the best application methods for growth factors for special indications. We have established that orthopaedic implants can be coated with growth factors and then serve as both a stabilisation device and a drug carrier with the potential to promote bone healing.

In this study we analysed the effect of locally applied PDGF on biodegradable PDLLA-coated implants on the healing of fractured tibiae in four-month-old Sprague-Dawley rats at three time points (three, seven and ten days) after the fracture. Only at the third time point (after ten days) was the distribution of proliferating cells in all cellular events higher than at the other two time points, which shows that PDGF stimulates osteoblasts and increases bone formation. With this technique, local application of PDGF from a biodegradable PDLLA-coated implant can accelerate fracture healing significantly.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References