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DELETION OF iNOS GENE IMPAIRES MOUSE FRACTURE HEALING

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Introduction:

Nitric oxide (NO) is a short-lived free radical gas produced from L-arginine by the nitric oxide synthases (NOS). There are three isoforms of NOS; two constitutive, endothelial (eNOS) and brain (bNOS) and one inducible (iNOS). All isoforms are expressed during rat fracture healing in a temporal fashion. iNOS is expressed earliest (peak day 4) among the three isoforms^{1,2}. The current study evaluated the role of iNOS on mice fracture healing by using a mid-shaft femoral fracture model in iNOS knockout (iNOS-KO) and wild mice.

Methods:

Animal model: Twelve weeks old female wild-type mice and iNOS-KO mice had a right femoral mid-shaft osteotomy fixed with a 0.5mm-diameter needle across the fracture site. A gelatine sponge (2mm diameter x 1mm thick) was implanted across the fracture site. Via a gelatine sponge either Ad5-CMVempty (adenovirus without gene) or Ad5-CMV*iNOS* (adenovirus with iNOS gene) was administered to the fracture site at a dose of 10⁷ pfu. Mice were divided into three groups: wild mice (n=12), iNOS-KO mice (n=12) with Ad5-CMVempty and iNOS-KO mice (n=14) with Ad5-CMV*iNOS*. Mice were sacrificed at day 14 and their right and left hind limbs harvested.

Cross sectional area (CSA): Callus CSA was determined by measuring the callus diameter across in the medio-lateral and antero-posterior plane using a vernier calliper. CSA of the un-operated left femur mid-shaft was determined as an internal control.

Biomechanics: Using a specialised jig, each end of the bone was centralised in concentric aluminium tubes (set apart by a constant distance of 5 mm) and fixed with PMMA cement. Specimen were gripped in a biaxial INSTRON testing system with consistent orientation, and loaded to failure torsionally. Maximum torque (Nm), torsional stiffness (Nm/deg), and total energy (Nm.deg) were determined from the torque-rotation plots.

Statistics: All data are presented as mean $\pm$ SE. Differences between experimental groups were assessed using analysis of variance (ANOVA), and Fisher's LSD post hoc test. The level of significance was set at $p < 0.05$.

Results:

Deletion of the iNOS gene did not affect the maximum torque, but decreased the total and maximum energy absorption by 30% and by 80% respectively ($p < 0.01$) in comparison to the wild mice healing femoral fracture (Table 1, Fig 1).

Table 1. Biomechanical properties of operated femora at day 14 shown as mean $\pm$ SE. iNOS-KO mice showed decreased total energy (**= $p < 0.01$) and rotation (*= $p < 0.05$).

Parameters	Wild type mice	iNOS KO mice	
	adenovirus administered n=12	adenovirus administered n=12	adenovirus <i>iNOS</i> transfected n=14
Total Energy x 10 ⁻³ (Nm.degrees)	99 $\pm$ 8	71 $\pm$ 6**	97 $\pm$ 7
Deformation (degrees)	57 $\pm$ 9	35 $\pm$ 3 *	54 $\pm$ 5
Stiffness x 10 ⁻⁵ (Nm / degree)	24 $\pm$ 3	29 $\pm$ 3	24 $\pm$ 2
Shear stress (MPa)	11 $\pm$ 1.8	9.1 $\pm$ 1.1	6.2 $\pm$ 1.3

This reduction in energy absorption was reversed by iNOS gene transfection in iNOS-KO mice. Furthermore, iNOS gene transfection

caused an increase in callus-CSA by 25% ($p < 0.01$), torsional failure by 20% ($p < 0.01$), total energy absorption by 40% ($p < 0.01$) and maximum energy absorption by 80% ($p < 0.01$) when compared to iNOS-KO mice (Fig 1, Table 1). There were no significant differences in the biomechanical properties of intact femora except for stiffness that was lower in wild mice by 45% in comparison to the iNOS-KO mice ($p < 0.05$). CSA of the wild mice intact femora was also smaller than iNOS-KO mice by 15% ($p < 0.001$).

Fig 1A. Maximum torque values of healing mice femora represented as mean and standard error (KO mice = iNOS knockout mice, **= $p < 0.01$).

Fig 1B. Maximum energy values of healing mice femora represented as mean and standard error (KO mice = iNOS knockout mice, **= $p < 0.01$).

Discussion:

Previous work has shown that iNOS is expressed in a temporal fashion during rat fracture healing with a peak intensity at day 4 post-fracture³. The work presented here shows that deletion of the iNOS gene decreased energy absorption in day 14 healing fractures. This effect was reversed by iNOS gene transfection. Local iNOS gene transfection increased callus cross-sectional area, maximum torque, and energy absorption at day 14 healing fracture when compared to the iNOS-KO mice. This data indicates that iNOS gene replacement reversed the detrimental effects of iNOS gene deletion confirming that iNOS is important in mouse fracture healing.

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Abstract

Nitric oxide (NO) is a signalling molecule synthesised from L-arginine by nitric oxide synthases (NOSs). NOS isoforms are either constitutively expressed (endothelial NOS [eNOS] and neuronal NOS [nNOS]) or inducible by lipopolysaccharides and pro-inflammatory cytokines (inducible NOS [iNOS]). Previously, our group has reported that NO is expressed during and modulates fracture healing. In this study we evaluated the specific contribution of iNOS to fracture healing by using iNOS gene therapy in iNOS-deficient mice. Twelve weeks old female wild-type mice and iNOS-KO mice had a right femoral mid-shaft osteotomy fixed with an intramedullary 0.5mm-diameter needle. A gelatine sponge was implanted across the fracture site. The gelatine sponge received either Ad5-CMV*iNOS* (in iNOS-deficient mice; n=16) or Ad5-CMVempty (in wild-type mice; n= 15 and, iNOS-deficient mice; n=15) at a dose of 10^7 pfu. Mice were sacrificed at day 14 and their right and left hind limbs harvested. Cross sectional area was determined by measuring the callus diameter across the medio-lateral and antero-posterior plane using a vernier calliper. Specimens were loaded to failure torsionally in a biaxial INSTRON testing system and maximum torque, torsional stiffness, and maximal and total energy determined.

Deletion of the iNOS gene decreased the total and maximum energy absorption of the healing femoral fracture by 30% and by 70% respectively ($p<0.01$) in comparison to the wild type mice. This reduction in energy absorption was reversed by iNOS gene transfection. Furthermore, iNOS gene transfection caused an increase in torsional failure by 20% ($p=0.01$) in comparison to iNOS(-/-) mice that did not receive the iNOS gene. There were no significant differences in the biomechanical properties of intact femora.

This data indicates that iNOS is important in mouse fracture healing. However, the clinical utility of NOS gene therapy to enhance fracture healing will need further evaluation.

Introduction

Nitric oxide (NO) is a short-lived free radical gas produced from L-arginine by the nitric oxide synthases (NOSs). NOS isoforms are either calcium dependent and constitutively expressed (cNOS) (e.g. neuronal NOS [nNOS] and endothelial NOS [eNOS]) or calcium independent inducible (iNOS). iNOS is expressed after exposure to diverse stimuli, such as inflammatory cytokines and lipopolysaccharide (LPS); once expressed, the inducible enzyme generates larger and more sustained amounts of NO than the constitutive isoforms (8, 13). Our Previous data indicate that NO plays a modulatory role in rat fracture healing (2, 3, 11).

A number of studies have indicated that iNOS is important in wound healing (15, 17-19). Yamasaki et al. (19) showed that wound healing was delayed in iNOS-deficient mice, and that this delay was completely reversed by a single application of an adenoviral vector containing human iNOS cDNA (AdiNOS) at the time of wounding. Thornton et al. (18) showed that iNOS is expressed during cutaneous wound repair and in vivo transfection of wound cells with the iNOS gene increased physiological wound NO levels and augment collagen accumulation. However, the role of iNOS in fracture healing has not been evaluated.

We have previously shown that all three isoforms of NOSs are expressed during rat and human fracture healing in a temporal and cell-specific fashion (11, 21, 22). However, the contribution of specific isoforms of NOS enzymes in fracture healing is not known. The current study evaluated the role of iNOS in mice fracture healing using a mid-shaft femoral fracture model in iNOS deficient [iNOS(-/-)] and wild-type [iNOS(+/-)] mice.

Materials and Methods

iNOS(+/-) and iNOS(-/-) mice embryos were provided by Dr. Guna Kampiah (University of Sydney, Australia) and bred at the Biologic Research Centre, University of New South Wales under the authorisation of their original developers Dr. John MacMicking and Dr. Carl Nathan (Cornell University, USA). Ad5-CMVempty and Ad5-CMV*iNOS* mouse gene constructs was purchased from Iowa University (Iowa, USA) and Gelfoam[®] absorbable gelatine sponge was purchased from, Pharmacia & Upjohn (Australia).

Animals and Experimental Design

Mice were housed under an artificial 12-hours day/ night light environment with 60% humidity at 21°C. A pilot study has been performed in order to establish operation technique. Twelve week old female iNOS(+/+) and iNOS(-/-) mice were anaesthetized with an intraperitoneal administration of sodium pentobarbital (40 mg/ kg body mass) and a right femoral mid-shaft osteotomy was made with a scalpel, the osteotomy fixed with an 0.5mm-diameter intramedullary needle. A gelatine sponge (2mm diameter x 1mm thick) was implanted across the fracture site at the time of operation. Either Ad5-CMVempty or Ad5-CMV*iNOS* (Ad5-CMV*iNOS* containing mouse *iNOS*cDNA) was administered to the fracture site at a dose of 10^7 pfu via the gelatine sponge. Mice were divided into three groups: iNOS(+/+) mice with Ad5-CMVempty (n=15), iNOS(-/-) mice with Ad5-CMVempty (n=15) and iNOS(-/-) mice with Ad5-CMV*iNOS* (n=16). Postoperative pain control was provided by a subcutaneous injection of 0.04 mg/kg of buprenorphine at the end of the operation. Mice were sacrificed at day 14 and both hind limbs harvested and stored in saline at -20°C. The Animals Ethics and Care Committee of the University of New South Wales approved all animal experiments.

Cross sectional area (CSA)

The femora of the disarticulated mouse limbs were dissected clean from the surrounding tissues. Callus diameter was measured in the antero-posterior and medio-lateral planes using a vernier calliper (Mitutoyo, Kawasaki, Japan). Cross sectional area SA was calculated by the formula $\pi r_1 r_2$ where r_1 is the radius in the sagittal plane and r_2 the radius in coronal plane followed by the subtraction of internal cross-sectional area of the medullary canal which was calculated by the formula πr^2 where r was the radius of intramedullary needle. Cross sectional area SA of the un-operated left femur mid-shaft was also determined as an internal control. Ratio of the callus cross sectional area CSA to that of un-operated left femur was also calculated. All femora were gauze wrapped and stored in saline at -20 °C prior to biomechanical evaluation.

Biomechanics

Using a V-notch guide, each end of the bone was centralised in two concentric aluminium tubes (set apart by a constant distance of 5 mm) and fixed with polymethyl methacrylate (PMMA) cement (Howmedica®, London, England). The specimens were gripped in a biaxial 8874 Instron testing system (Instron Co, England) with consistent orientation and loaded torsionally to failure at a rotational rate of 10 degrees/ second. Torque, rotation and time were collected. Maximum torque (Nm), torsional stiffness (Nm / degree), and maximal and total energy

(Nm x degree) were determined from the torque-rotation plots. Modulus of rigidity (G) was calculated using the equation derived from Roark(20).

Statistics: All data were presented as mean $\pm$ SE. Differences between experimental groups were assessed using analysis of variance (ANOVA), and LSD post hoc test. The level of significance was set at $p < 0.05$.

Results

Body weight gain

Average iNOS(-/-) mice body weight were heavier when compared to wild type mice of the same age by 15% and 10% before and after operation respectively ($p < 0.001$). At day 14 after operation wild-type mice and iNOS(-/-) mice that received the iNOS gene gained 5% of previous body weight while iNOS(-/-) mice that did not receive iNOS gene, did not gain weight ($p < 0.01$).

Callus morphology

There was a significant difference in the CSA cross sectional area of the intact left femora between groups by 20% ($p < 0.001$) (Fig.1). On the operated side, callus CSA cross sectional area was greater in the iNOS(-/-) mice with Ad5-CMV*iNOS* than that of wild-type mice and iNOS(-/-) mice by 30% ($p < 0.001$) and 10% ($p < 0.01$) respectively (Fig.1).

The ratio of the callus CSA cross sectional area to that of un-operated left femur was 8.7 in wild-type mice, 8.2 in the iNOS(-/-) mice and, 8.9 in iNOS(-/-) mice with Ad5-CMV*iNOS*, and there were no statistically significant differences in the ratio of CSA cross sectional area.

Biomechanical analysis

Deletion of the iNOS gene decreased the total and maximum energy absorption of the healing femoral fracture by 30% ($p = 0.01$) and 70% ($p < 0.01$) respectively in comparison to the wild type mice. This reduction in energy absorption was reversed by iNOS gene transfection in iNOS(-/-) mice (Table 1, Fig 2B). Furthermore, iNOS gene transfection caused an increase in torsional failure by 20% ($p = 0.01$), total energy absorption by 30% ($p < 0.05$) and maximum energy absorption by 80% ($p < 0.01$) when compared to iNOS(-/-) mice (Fig 2A-B, Table 1). There were no significant differences in the biomechanical properties of intact femora between groups.

Discussion

The current work showed that deletion of the iNOS gene decreased maximum energy absorption by 70% at day 14 healing femoral fracture in mice. This effect was reversed by iNOS gene transfection. Local iNOS gene administration in iNOS(-/-) mice increased callus CSA, maximum torque, and energy absorption when compared to the iNOS(-/-) mice that did not receive iNOS gene.

We have noted that at the same age, average iNOS(-/-) mice body weight ~~was greater~~ were heavier than wild-type mice suggesting that iNOS may also have effect on energy metabolism. At day 14 after operation wild-type mice and iNOS(-/-) mice that received iNOS gene ~~gained~~ were 5% of previous body weight, but, heavier than before their operation, while mice lacking the iNOS gene failed to gain weight, after operation ~~which may be due to poor healing response and recovery.~~ We speculate that this inability to gain weight in the iNOS deficient mice may be due to a poorer healing response and slower recovery.

~~CSA of intact femur showed significant difference between groups, with the wild-type mice having the smallest CSA. However the biomechanical properties of the intact femur did not differ between groups.~~ The intact femora were smaller but just as strong as the other groups.

We have previously shown that competitive inhibition of nitric oxide synthase activity inhibits fracture healing and that supplementation of NO can reverse this effect (11). We have also shown that deficits in the rat femur can fill in more rapidly when supplemented by an NO donor These data suggest that NO is an important mediator of fracture healing. ~~CSA of the callus was greater in iNOS(-/-) that received the iNOS gene in comparison to the wild-type mice and iNOS(-/-) mice. However when the ratio of the callus CSA to the that of contralateral intact femur was compared there was no significant difference between groups.~~

~~Previous data has indicated that pharmacological inhibition of NOSs had a detrimental effect on fracture healing that can be reversed by local NO supplementation in rat fracture model (11). Local NO supplementation by using S-nitrosothiol compounds can improve gap filling rate in critical size rat femoral defect model(3) and biomechanical properties of healing femora in rat fracture model(2). These evidences suggest that NO is an important mediator of fracture healing.~~

There is no messenger RNA(mRNA) expression, immunoreactivity, or enzymatic activity for NOS in unfractured femoral cortex, however, after fracture mRNA, protein, and enzymatic activity for iNOS were

identified in the healing rat femoral fracture callus, with maximum activity at day 4 (11, 21). The initial stage of fracture healing includes a hematoma formation and inflammation (12). ~~Evidences suggest~~ It is likely that signalling molecules that are released during the initial phase have the capacity to initiate the cascade of cellular events that are critical to fracture healing (7, 9, 12, 21, 22). iNOS is expressed with maximum activity during the initial inflammatory phase of fracture healing, ~~thus, We speculate that iNOS plays a modulatory role during the initial fracture healing phase, probably as a component of inflammatory response to the injury.~~

~~Adenovirus~~ Adenoviral vectors associated gene delivery with osteoinductive application has been shown to be promising in preclinical models have been successfully used to deliver genes and induce bone formation (1, 4-6, 14, 16) (14, 16). Baltzer et al directly injected an adenovirus containing the cDNA for BMP-2 (Ad.BMP-2) into a critical-sized defect in a rabbit femur (4, 5). ~~Recently, our group reported that~~ We have transfected primary cultured human rotator cuff tendon cells and rat Achilles healing tendon in-vivo ~~can be transfected with~~ by Ad5CMVntLacZ, an adenovirus containing the reporter gene LacZ, in a dose dependent manner without impairing cell viability. The duration of LacZ expression in vivo was 17 days. Transfection efficiency was enhanced when a gelatin sponge was used to deliver the adenovirus (10).

The data presented ~~data in this paper~~ indicates that an adenovirus ~~adenoviral~~ vector associated ~~can replace the~~ iNOS and gene replacement reversed ~~reverse~~ the detrimental effects of iNOS gene deletion on fracture healing as indicated ~~mainly by biomechanical data confirming that~~. This work also confirms that iNOS is important in mouse fracture healing. ~~iNOS gene therapy using adenovirus vectors may be a promising therapeutic approach for the clinical conditions associated with delayed fracture healing and non-unions. However, mechanism of iNOS action, the contribution and interaction of the other NOS isoforms remains unknown and requires more detailed investigation.~~

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