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Sequential induction of pro- and anti-inflammatory prostaglandins and peroxisome proliferators activated receptor-gamma during normal wound healing: A time course study.

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Summary

Lipid mediators generated from metabolism of arachidonic acid play a crucial role in the initiating and resolution of acute inflammation by shifting from pro-inflammatory prostaglandin (PG) E₂ to anti-inflammatory PGD₂ and its metabolites. The changes in PG levels over time during the normal wound repair process have not, however, been reported. We determined the temporal expression of PG and their biosynthetic enzymes using the full thickness incisional model of normal wound healing in mice. We demonstrate that during normal wound repair, there is a shift in the metabolism of arachidonate from PGE₂ during the acute inflammatory phase to PGD₂ during the repair phase. This shift is mediated by temporal changes in the expression of cyclooxygenases (COX) and microsomal PGES (mPGES)-1. Inducible COX (COX-2) expression is sustained throughout the initiation and repair process, but mPGES-1 is increased only during the acute inflammatory phase and its disappearance coincides with increased PGD₂. PGD₂ and its degradation products are known to mediate their anti-inflammatory effects by binding to peroxisome proliferators activated receptor gamma (PPAR γ). In this study, we show that PPAR γ is upregulated during the resolution phase of wound repair concomitant with the shift to PGD₂, and may be responsible for initiating endogenous mechanism resulting in healing/resolution.

Introduction

Normal wound repair is a multifactorial pathophysiological process involving complex interactions of soluble mediators, growth factors and cytokines which help repair the injured tissue [1]. Extensive work has been carried out in order to elucidate the underlying mechanisms which initiate the acute inflammatory response in a wound repair process, but little attention has been focused towards the endogenous mediators which may help resolve the inflammatory episode during the time course of a normal wound healing process. Recent studies in other models of acute inflammation have indicated that prostaglandins (PGs) generated from the metabolism of arachidonic acid (AA) may play a crucial role in not only initiating the inflammation but also in the resolution of inflammation. The PG profile in these models shifts from the predominantly pro-inflammatory prostaglandin (PG)E₂ to the anti-inflammatory PGD₂ during the time course of acute inflammation [2–4]. This temporal pattern of AA

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metabolism during the time course of normal wound repair process has not previously been reported.

During tissue injury, PGs are formed by metabolism of AA by cyclooxygenases (COX) to generate an intermediate substrate PGH_2 which is further metabolized by terminal synthases to generate specific PGs [4–6]. These terminal synthases include PGE synthases (PGES) for PGE_2 , PGDS for PGD_2 , PGFS for $\text{PGF}_{2\alpha}$ and PGIS for PGI_2 (prostacyclin) respectively [7]. COX exists in two isoforms, COX-1 and COX-2. A splice variant of COX-1 has also been reported in some tissues but its biological relevance is unclear [8,9],[10]. During wound repair, COX-1 is constitutively expressed while elevated levels of COX-2 are observed during the early inflammatory phase of wound repair [11]. However, recent studies in animal models of acute inflammation suggest that COX-2 and COX-2 derived PGs may have anti-inflammatory properties [2,3]. The majority of the wound healing studies that have focused on PG metabolism have explored only the pro-inflammatory role of COX-2. No report to date has evaluated the potential role of COX-2 and its derived PGs towards resolution of inflammation in a normal wound repair process.

Among terminal PG synthases, PGES specifically catalyzes the conversion of PGH_2 to PGE_2 [11–13]. At least three forms of PGES have been cloned and characterized, and are termed as cytosolic PGES (cPGES), microsomal PGES (mPGES)-1, and mPGES-2 [12–14]. mPGES-1 is a glutathione-dependent enzyme that shows coordinated induction with COX-2 by inflammatory stimuli in various cells and tissues [15,16]. Since it is an inducible enzyme, it has been of great interest to investigate the role of mPGES-1 in inflammatory processes. cPGES is constitutively expressed in the cytosol and is functionally coupled with COX-1 under basal conditions [13]. mPGES-2 is also constitutively expressed in various cells and tissues [17]. No report has elucidated the contribution of mPGES-1, cPGES and mPGES-2 towards the process of normal wound healing.

PGDS is the key terminal synthase involved in the conversion of PGH_2 to PGD_2 . Two distinct forms of PGDS, lipocalin PGDS (L-PGDS) and hematopoietic PGDS (H-PGDS) have been cloned and characterized [18,19]. L-PGDS is mainly involved in central nervous system including sleep induction, body temperature, and analgesia [18]. H-PGDS is widely distributed in peripheral tissues [19] and is a key enzyme for the readily undergoes dehydration *in vitro* and *in vivo* to yield production of PGD_2 . PGD_2 biologically active PGs of the J_2 series [20, 21]. J_2 series PGs are characterized by the presence of a reactive, β -unsaturated ketone in the cyclopentenone ring and therefore referred as cyclopentenone PGs [22]. Cyclopentenone PGs are actively incorporated into cells and accumulate in the nucleus and form adducts with various proteins and transcription factors [23]. PGD_2 and its J-series degradation product, 15-deoxy- $\Delta^{12,14}$ - $\text{P}_{\text{J}}\text{G}_2$ (15dPGJ₂) have been shown to exert anti-inflammatory effects on peripheral inflammation and promote resolution via inhibition of various pro-inflammatory signaling molecules including cytokines, inducible nitric oxide synthase (iNOS) and nuclear factor kappa B (NF κ B) [2,24–30]. The majority of the anti-inflammatory mechanisms mediated by PGD_2 and its dehydrated product 15dPGJ₂ are now thought to be mediated by peroxisome proliferator activated receptor gamma (PPAR γ) [31]. This is based on the fact that 15dPGJ₂ is an endogenous ligand for PPAR γ and its activation results in upregulation of mechanisms involved in the resolution of inflammation [27,28,31,32]. *In vitro* and *in vivo* studies using endogenous and synthetic ligands of PPAR γ have been shown to inhibit broad range of pro-inflammatory immune mediators including interleukin (IL)-1 β , tumour necrosis factor (TNF)- α and NF κ B [33,34]. Animal studies using rosiglitazone (a potent synthetic ligand of PPAR γ) also suggest that rosiglitazone has the ability to limit inflammation through a PPAR γ dependent pathway [35–37]. No report has demonstrated the role of H-PGDS, PGD_2 and PPAR γ in the resolution of a normal wound healing process.

We hypothesized that analogous to other inflammatory processes, the initiation and resolution phases of normal wound healing would be associated with characteristic patterns of biosynthetic enzymes and product expression. Since it is likely that anti-inflammatory mechanisms result from the engagement of PGs such as PGD₂ with PPAR γ and its downstream targets, we studied PPAR γ in parallel. To elucidate the role of these endogenous mediators during the process of normal wound repair, it was vital to determine their temporal profiles during the complete time course of a normal wound repair. The aims of this study were to determine the temporal expression profiles of COX-1, COX-2, mPGES-1, mPGES-2, cPGES, hPGDS, PGIS; temporal production profiles of major AA metabolites including PGE₂, PGD₂, 6-keto-PGF_{1 α} ; and the temporal expression profile of PPAR γ during the inflammatory and resolution phases of normal wound healing process. To address these questions we used a well established full thickness incisional model of normal wound healing in mice.

Materials and Methods

Materials

Female DBA/1 Lac J mice were purchased from Jackson laboratories (Bar Harbor, Maine), Rabbit anti-human mPGES-1 antiserum was gifted from Dr. Per-Johan Jakobsson (Karolinska Institute, Stockholm). Rabbit anti-mouse COX-2 polyclonal antibody, rabbit anti-human mPGES-2 polyclonal antibody, rabbit anti-human cPGES polyclonal antibody, rabbit anti-mouse PGI synthase (PGIS) polyclonal antibody, rabbit anti-mouse hematopoietic PGD synthase (H-PGDS) polyclonal antibody, rabbit anti-mouse COX-1 polyclonal antibody, enzyme-linked immunosorbent assay (ELISA) kits for PGE₂, 6-keto-PGF_{1 α} and PGD₂ were all purchased from Cayman Chemical Co. (Ann Arbor, MI). Mouse monoclonal PPAR γ primary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human beta-actin monoclonal antibody was obtained from Sigma Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were obtained from Jackson ImmunoResearch (West Grove, PA). TRIPure was purchased from Roche Diagnostics (Indianapolis, IN). The polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence (ECL) reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Animals

Female DBA/1 Lac J mice (25–30g) were housed at 25°C and communally kept on a 14 hour light/10h dark cycle. Standard mouse diet and water were available *ad libitum*. All mice were allowed to acclimatize for at least three days before shaving/wounding. Following surgery, animals were housed individually at 25°C and kept on a 14 hour light/10h dark cycle.

Wound model

Twenty four hours prior to wounding, animals were anaesthetized with ketamine 80–100 mg/kg and xylazine 8–10 mg/kg (mixed) injected IP using ½ inch 26 gauge needles, the backs were shaved and incision points marked on the dorsum. Twenty four hours after shaving the animals were anaesthetized again and 1cm full dermal thickness incisional wounds were made 3 and 8 cm (using a sterile surgical scalpel blade) from the base of the neck and 2 cm from the spine to give a total of 4 wounds per animal. Wounds were left uncovered; the animals were allowed to recover from anesthesia and then housed individually.

Tissue collection

Immediately after euthanasia, the tissue samples were harvested from the wounded regions of the mice on days 0, 1, 3, 7, 14 and 21, and were subsequently used to perform

immunohistochemistry, western blotting, reverse transcription-polymerase chain reaction (RT-PCR) and enzyme immunoassays (EIA).

Reverse transcription-polymerase chain reaction

RNA from the isolated wound tissues was extracted with TRIpure reagent according to the manufacturer's instructions. Reverse transcription was performed according to the manufacturer's instructions using a SuperScript preamplification system (Invitrogen, Carlsbad, CA) with 1 µg of total RNA from the tissues as a template. Subsequent amplifications of the cDNA fragments by PCR with HotStarTaq polymerase (Qiagen, Valencia, CA) were performed using 0.5 µl of the reverse-transcribed mixture as a template with specific oligonucleotide primers and cycle number as follows: mouse mPGES-1 (28 cycles), sense 5'-CAC ACT GCT GGT CAT CAA GA-3' and antisense 5'-ACA CCA AGT CCG CAA GTT C-3'; mouse COX-2 (25 cycles), sense 5'-GGG CCC TTC CTC CAG TAG CAG A-3' and antisense 5'-CAT CAG ACC AGG CAC CAG ACC AA-3'; mouse mPGES-2 (25 cycles), sense 5'-GGT GGC CCA GGA AGG AGA CAG C-3' and antisense 5'-GCA GCC GCG CCC ACA TAC TTG-3'; mouse cPGES (30 cycles), sense 5'-CCC GCC CAC CCG TTT GTC-3' and antisense 5'-TCT GGC ATC TTT TCA TCA TCA CTG-3'; mouse PGIS (30 cycles), sense 5'-TTC TGG CTC CTT CTT TTC CTC CTC-3' and antisense 5'-CTT CAG CCG TTT CCC ATC TTT GTA -3'; mouse H-PGDS (31 cycles), sense 5'-ATG CCT AAC TAC AAA CTG CTT-3' and antisense 5'-CTA GAG TTT TGT CTG TGG CCT-3'; mouse COX-1 (30 cycles), sense 5'-CCC CAG CCC TCC GAC CTA CAA-3' and antisense 5'-CCC CGG AAG CAA CCC AAA CAC-3'; mouse PPAR γ (31 cycles), sense 5'-CCT CTC CGT GAT GGA AGA CC-3' and antisense 5'-GCA TTG TGA GAC ATC CCC AC-3'; mouse GAPDH (20 cycles), sense 5'-GGG GTG AGG CCG GTG CTG AGT AT-3' and antisense 5'-CAT TGG GGG TAG GAA CAC GGA AGG-3'. After initial denaturation at 95°C for 15 min, PCR involved amplification cycles of 30 sec at 95°C, 30 sec at 56°C and 45 sec at 72°C, followed by elongation for 5 min at 72°C. The amplified cDNA fragments were resolved by electrophoresis on 2% (w/v) agarose gel and were visualized under UV light using a BioRad Chemidoc Apparatus (Hercules, CA) after staining of the gel with ethidium bromide.

Western blotting

Wound tissues were homogenized in 50 mM Tris-buffered saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS) and the protein content was determined using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard. Wound homogenates were adjusted to equal equivalents of protein and then were applied to SDS-polyacrylamide gels (10–20%) for electrophoresis. Next, the proteins were electroblotted onto polyvinylidene difluoride membranes. After the membranes were blocked in 10 mM TBS containing 0.1% Tween-20 (TBS-T) and 5% skim milk, the membranes were probed for 1.5 hrs with the respective antibodies (1:1000 for mPGES-1, COX-2, mPGES-2, cPGES, PGIS, H-PGDS, COX-1, PPAR γ and beta-actin) in TBS-T for 1.5 hrs. After washing the membranes with TBS-T, the membranes were incubated with HRP-conjugated anti-rabbit (for mPGES-1, COX-2, mPGES-2, cPGES, PGIS, H-PGDS and COX-1) or HRP-conjugated anti-mouse (for PPAR γ and beta-actin) IgG (1:10,000 dilution in TBS-T containing 5% skim milk) for overnight at 4°C. After further washing with TBS-T, protein bands were visualized with an ECL Western blot analysis system using a BioRad Chemidoc Apparatus (BioRad, Hercules, CA).

Enzyme Immunoassay

The concentrations of PGE₂, 6-keto-PGF_{1 α} (a stable metabolite of PGI₂) and PGD₂ in the wounded skin tissue were measured by ELISA (Cayman Chemicals, Ann Arbor MI). Assays were performed according to the manufacturer's recommendation.

Immunohistochemistry

Frozen wound tissues were embedded in optimal cutting temperature (OCT) compound and 0.5 μ m sections were cut using a cryostat (Leica, Bannockburn, IL) and collected on poly-L-lysine-coated slides. Immunolabeling of COX-2, mPGES-1 and PPAR γ were performed using the DakoCytomation LSAB+ System-HRP kit. Immunohistochemical procedures were performed according to the manufacturer's recommendations. Briefly, endogenous peroxidase was blocked using 0.5% H₂O₂ in methanol for 5 minutes. Non-specific IgG binding was blocked by incubating sections with bovine serum albumin (0.1%) in PBS for 1 hour and then incubated with primary antibodies for COX-2, mPGES-1 and PPAR γ (1:100) in a humidified chamber and left overnight at 4°C. Next, sections were incubated with biotinylated link for 30 minutes followed by incubation with streptavidin for 30 minutes. The chromogen diaminobenzidine tetrahydrochloride (DAB), was then added till sufficient color development and sections counterstained with Harris's hematoxylin.

Statistical analysis

The data are expressed as mean $\pm$ SEM. Statistical analysis was performed using Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Differential induction pattern of COX-2 and mPGES-1 during inflammatory phase (days 1 and 3) and resolution phase (days 7, 14 and 21) of normal wound healing

Protein and mRNA expression profiles of COX-2 and mPGES-1 were determined by western blotting and RT-PCR, respectively. Our results show that, COX-2 and mPGES-1 were co-induced during early inflammatory phase (days 1 and 3 postwounding) of wound healing process. Peak protein (Fig. 1) and mRNA (Fig. 2) expression levels for both COX-2 and mPGES-1 were detected on day 3 postwounding ($P < 0.05$). However, during the resolution phase of normal wound repair process, a differential expression profile for COX-2 and mPGES-1 was detected. Following peak on day 3 postwounding, mPGES-1 protein and mRNA expression levels rapidly depleted and were undetectable on day 21 postwounding. For COX-2, the expression levels (mRNA and protein) on day 7 reduced to a lesser extent compared to day 3 postwounding and sustained expression levels for COX-2 were maintained till day 21 postwounding ($P < 0.05$ for days 7, 14 and 21). Thus, COX-2 in comparison to mPGES-1 was upregulated not only during inflammatory phase but also during the resolution phase of wound healing process.

Localization of COX-2 and mPGES-1 during normal wound repair process

COX-2 and mPGES-1 localization in the wound tissue was detected by immunohistochemistry. Results show that peak expression levels of COX-2 (Fig. 3a) and mPGES-1 (Fig. 3b) were observed on day 3 postwounding in concordance with the protein and mRNA expression profiles. Immunohistochemical staining of wound sections showed that both COX-2 and mPGES-1 were expressed in the epidermal and dermal regions of the wounded skin. However, COX-2 expression levels were most intense in the dermis area at day 3 postwounding, whereas lower levels of COX-2 were observed in the epidermal region of the wounded skin. Contrastingly, high levels of mPGES-1 were detected in the epidermis of day 3 wound sections whereas low levels were detected in the dermis region of the wounded skin. We also determined the localization of H-PGDS in days 3 and 7 wounds. However, we were unable to localize H-PGDS in the epidermal and dermal regions of the wounded skin (data not shown). This may be due to very low levels of H-PGDS expressed during wound repair response.

Constitutive expression of COX-1, mPGES-2, cPGES, H-PGDS and PGIS during the inflammatory and resolution phases of normal wound healing process

Our results show that COX-1, mPGES-2, cPGES, H-PGDS and PGIS protein (Fig. 4a) and mRNA (Fig. 4b) expressions were detectable on day 0 (unwounded skin), suggesting the presence of these enzymes in the skin under unwounded state. However, no change in the expression levels of COX-1, mPGES-2, cPGES, H-PGDS and PGIS were detected during early inflammatory as well as the resolution phases of normal wound healing process. Thus, these enzymes were constitutively expressed throughout the time course of during normal wound repair process.

Differential production pattern of PGE₂ and PGD₂ during inflammatory and resolution phases of wound healing process

Levels of PGE₂ and PGD₂ (Fig 5) in the wound tissues were determined by EIAs. Low levels of PGE₂ were detected in the day 0 unwounded control skin. A significant increase in the levels of PGE₂ were observed on day 1 ($P<0.05$) and 3 ($P<0.05$) postwounding, peaking on day 3 and subsequently levels returned to normal basal levels by days 7, 14 and 21 postwounding.

Low levels of PGD₂ were observed on day 0 and day 1 postwounding. However, a significant increase in the levels of PGD₂ were observed on 7 ($P<0.05$), 14 ($P<0.05$) and 21 ($P<0.05$) postwounding, peaking on day 7. We also determined the temporal production levels of 6-keto-PGF_{1 α} during the time course of wound repair. Very low basal levels of 6-keto-PGF_{1 α} were observed before wounding and during the complete time course of normal wound repair process (data not shown). Thus, PGE₂ and PGD₂ production profiles showed a differential production pattern with peak levels of PGE₂ observed during early inflammatory phase of normal wound repair process. However during the resolution phase of normal wound repair, there was a switch in the metabolism towards PGD₂ which was the predominant PG product.

Increased expression of PPAR γ during the resolution phase of normal wound repair process

PPAR γ expression (protein and mRNA) were detectable in day 0 unwounded control skin (Fig 6a and 6b). Low levels of PPAR γ (protein and mRNA) were observed during early inflammatory phase of wound repair process. However, a significant increase in the expression levels of PPAR γ both at protein and mRNA levels were observed during the resolution phase (day 7, 14 and 21 postwounding), peaking at day 14 ($P<0.05$) for protein expression and day 21 ($P<0.05$) for mRNA levels. Immunohistochemistry results on day 14 wound sections show that PPAR γ was predominantly expressed in the epidermal region of the wounded tissue whereas low levels of PPAR γ were detected in the dermis (Fig 6c).

Discussion

This study for the first time reports a distinct temporal pattern of AA-derived PGs during inflammatory and resolution phases of normal wound repair. Using a well established full thickness incisional model of normal wound healing in mice we show that during inflammatory phase of wound repair, PGE₂ is the predominant AA-derived metabolite. However, as the inflammation subsides and wound repair process approaches resolution there is a switch in the AA metabolism from pro-inflammatory PGE₂ towards anti-inflammatory PGD₂. This temporal switch may play a key role in initiating early inflammation and subsequent transition towards resolution of inflammation during a normal wound repair process.

Vast literature suggests that PGE₂ is a major pro-inflammatory PG product produced during inflammation though some anti-inflammatory and immunosuppressive effects of PGE₂ have also been reported [8,38]. We and others have previously shown that COX-2 and mPGES-1 are key enzymes involved in the production of PGE₂ during inflammation [11,39,40]. In the

present study we demonstrate that mPGES-1 along with COX-2 are upregulated during early inflammatory phase of wound repair process with no change in the levels of other metabolic enzymes including COX-1, mPGES-2, cPGES, H-PGDS and PGIS. The expression of mPGES-1 drops rapidly to basal levels during the resolution phase; however, expression levels of COX-2 remain elevated till day 21 postwounding. Interestingly, levels of PGE₂ mirrored the temporal expression profile of mPGES-1 exhibiting elevation in the levels upon wounding with a peak on day 3 and thereafter returning to baseline during the beginning of resolution phase of wound repair. This confirms previous observations that production of PGE₂ during inflammation requires induction of COX-2 and mPGES-1 [11,39,40]. Recently, using embryonic fibroblasts isolated from mice deficient with mPGES-1, we reported that mPGES-1 is essential for induced PGE₂ production [40]. Results presented in this study also suggest that increased mPGES-1 expression along with induction of COX-2 coincide with the high levels of PGE₂ produced during wound repair. However, there remains the possibility of involvement of COX-1 for the generation of PGE₂ during wound repair. The fact that COX-2 is highly expressed in the dermis while mPGES-1 is highly expressed in the epidermis suggests that COX-1 could be a source for PGH₂ used by mPGES-1 to generate PGE₂ during wound repair. A previous study showed a tight coupling of COX-1 expression with PGE₂ and PGD₂ biosynthesis in the region of wounds instead of COX-2 during wound repair [41]. However, this study used a full thickness excisional wound healing model in BALB/C and C57BLKS mice in comparison to full thickness incisional wound healing model used in the present study using DBA/1 Lac J mice.

In this study we also observed that when PGE₂ levels were elevated during early inflammatory phase of wound repair, PGD₂ levels were low. Simultaneously, as the PGE₂ levels dropped, there was a significant elevation in the levels of PGD₂ which remained elevated during the resolution phase of normal wound healing process. This transition in the PG production pattern during the resolution phase of wound repair may in part be due to the ability of COX enzymes to convert AA to PGH₂ and subsequently PGD₂ in the absence of mPGES-1. It also vital to note that no change in the levels of H-PGDS were observed during inflammatory and resolution phases of wound healing process but constitutive H-PGDS levels may in fact be sufficient to convert PGH₂ to PGD₂ in the absence of mPGES-1.

Upregulation of PGD₂ during the resolution phase of wound healing process observed in this study provides further support for recently reported anti-inflammatory/resolution mechanisms mediated by PGD₂ [2,8,24,25,30,42]. PGD₂ and its degradation product, 15dPGJ₂ exhibit anti-inflammatory effects and induce crucial events in the resolution of inflammation, including inhibition of cytokines such as TNF- α , IL-1 β and IL-6 and inhibition of NF- κ B [5,19–25,34]. More recently, PGD₂ and its degradation product 15dPGJ₂ were shown to initiate resolution of acute inflammation in rat pleurisy by causing neutrophil and macrophage apoptosis [43]. On the contrary, studies have also reported pro-inflammatory effects associated with PGD₂. PGD₂ has been shown to play a key pro-inflammatory role in allergic diseases such as allergic rhinitis and asthma [44]. Antagonists of the PGD₂ receptors have also shown promise for the treatment of asthma and rhinitis [45].

Recent studies suggest that the mechanisms by which PGD₂ and its metabolites mediate their anti-inflammatory effects are due in part to their action as PPAR γ ligands. We therefore determined the temporal profile of PPAR γ during the complete time course of normal wound healing. Our results show that during the resolution phase of wound repair a significant increase in the expression of PPAR γ mirrored the temporal profile of PGD₂. Furthermore, using immunohistochemical technique we show that PPAR γ expression was predominantly localized in the epidermal region of the wounded skin. This is the first report demonstrating that PPAR γ is upregulated during the resolution phase of wound repair. Based on the fact that PPAR γ expression mirrored the profile of PGD₂ during wound repair process, and that

PGD₂-derived metabolites are important PPAR γ ligands, we hypothesize that PPAR γ and PGD₂ may cooperate to achieve resolution of inflammation during wound repair.

In summary, we have shown that during the time course of wound repair, there is a gradual shift in the metabolism of AA from pro-inflammatory PGE₂ (during inflammatory phase) to anti-inflammatory PGD₂ (during the resolution phase). This shift in the PG production profile may in part be regulated by temporal changes in the expression levels of mPGES-1 enzyme and COX-2 during early inflammatory and resolution phases of wound repair. This study further shows the contribution of COX-2 may not be limited to the early inflammation associated with wounding and may well contribute to the resolution of inflammation by generating PGD₂ during later stages of repair. In addition, we show that PPAR γ is upregulated during the resolution phase of wound repair process concomitant with PGD₂ and may in part be responsible for initiating endogenous mechanism resulting in healing/resolution.

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Figure 1. Temporal protein expression profile of COX-2 and mPGES-1 during the time course of normal wound healing

Protein expressions of COX-2 and mPGES-1 in the isolated wound tissues were determined by western blotting during a complete time course of 21 days comprising of: day 0 (Control unwounded skin), days 1 and 3 (inflammatory phase) and days 7, 14 and 21 (resolution phase). COX-2 and mPGES-1 proteins were normalized by β -actin which was used as an internal control to assess equal protein loading. * indicates statistical significance at $P < 0.05$ at each respective time point compared to unstimulated control skin on day 0. Data are expressed as the mean $\pm$ SEM for $n=4$ separate blots.

Figure 2. Temporal mRNA expression profile of COX-2 and mPGES-1 during the time course of normal wound healing

mRNA expressions of COX-2 and mPGES-1 in the isolated wound tissues were determined by RT-PCR during a complete time course of 21 days comprising of: day 0 (Control unwounded skin), days 1 and 3 (inflammatory phase) and days 7, 14 and 21 (resolution phase). COX-2 (25 cycles) and mPGES-1 (30 cycles) mRNA levels were normalized by GAPDH (20 cycles) which was used as an internal control. * indicates statistical significance at $P < 0.05$ at each respective time point compared to unstimulated control skin on day 0. Data are expressed as the mean $\pm$ SEM for $n=4$ separate gels.

Figure 3. Localization of COX-2 and mPGES-1 in the day 3 wounds

COX-2 and mPGES-1 localization in the day 3 wound tissue was detected by immunohistochemistry. Fig 3a shows extensive staining of COX-2 in the dermal region of the wounded skin in the day 3 wound tissues. Fig 3b shows extensive staining of mPGES-1 in the epidermal region of the wounded skin in the day 3 wound tissues. WS= wound site, D=dermis, E=epidermis. → signifies the region of extensive staining. Magnification x10.

Figure 4. Temporal protein (a) and mRNA (b) expression profiles of COX-1, mPGES-2, cPGES, H-PGDS and PGIS during the time course of normal wound healing
 (a) Protein and (b) mRNA expressions of COX-1, mPGES-2, cPGES, H-PGDS and PGIS were determined by western blotting and RT-PCR respectively during a complete time course of 21 days comprising of: day 0 (Control unwounded skin), days 1 and 3 (inflammatory phase) and days 7, 14 and 21 (resolution phase). Proteins were normalized by β -actin (a) and mRNA expressions were normalized with GAPDH (b). Representative data for n=3 separate blots and gels is shown.

Figure 5. Temporal production profile of PGE₂ and PGD₂ during the time course of normal wound healing

The levels of PGE₂ (light bars) and PGD₂ (dark bars) in the wound tissues were determined by EIAs during a complete time course of 21 days comprising of: day 0 (Control unwounded skin), days 1 and 3 (inflammatory phase) and days 7, 14 and 21 (resolution phase). Levels of PGs were normalized to mg of protein for each respective wound tissue. * indicates statistical significance at $P < 0.05$ at each respective time point compared to unstimulated control skin on day 0. Data are expressed as the mean $\pm$ SEM for $n=7$ separate determinations.

(a)

(b)

(c)

PPAR γ **Figure 6. Temporal expression of PPAR γ during the time course of normal wound healing**

(a) Protein and (b) mRNA expressions of PPAR γ were determined by western blotting and RT-PCR respectively during a complete time course of 21 days comprising of: day 0 (Control unwounded skin), days 1 and 3 (inflammatory phase) and days 7, 14 and 21 (resolution phase). PPAR γ protein (a) and mRNA (b) expressions at each respective time points were normalized by β -actin and GAPDH respectively. * indicates statistical significance at $P < 0.05$ at each respective time point compared to unstimulated control skin on day 0. Data are expressed as the mean $\pm$ SEM for $n=4$ separate blots. (c) PPAR γ localization in the day 3 wound tissues was detected by immunohistochemistry. Extensive staining of PPAR γ was observed in the epidermal region of the wounded skin in the day 14 wound tissues. WS= wound site, D=dermis, E=epidermis. $\rightarrow$ signifies the region of extensive staining. Magnification $\times 10$.