In vitro and *in vivo* characteristics of frozen/thawed neonatal pig split-skin strips: A novel biologically active dressing for areas of severe, acute or chronic skin loss

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Abstract. The recurrent shortage of human skin autografts or allografts used to close extensive wounds has rekindled the search for feasible alternatives. In the past, adult pig skin was a popular biological dressing, yet doubts regarding its benefits have induced most people to abandon its use. Here we investigated the aptness of neonatal pig split-skin (NPSS) strips to be used as a novel kind of temporary dressing for areas of skin loss. NPSS strips are able to be prepared in bulk amounts, stored at -80˚C for up to six months, and recovered by swift thawing at 45˚C with no change in histological structure. When set into organ cultures *in vitro* for up to three weeks, these frozen/thawed NPSS strips exhibited both a skin-typical energy-linked metabolism (i.e., a predominant consumption of L-glutamine instead of glucose), and an enduring ability to secrete cytokines/chemokines such as IL-1α, IL-6, GM-CSF, and TNF-α; all features alike in quantitative terms to those exhibited by freshly prepared NPSS strips directly set into culture. Moreover, once applied as temporary dressings onto deep burn wounds *in vivo*, frozen/thawed NPSS strips produced, for at least seven days, porcine IL-1α, IL-6, GM-CSF, TNF-α, and TGF-ß; the cytokines/chemokines importantly involved in wound healing. Hence, frozen/thawed NPSS strips not only are capable of closing extensive areas of skin loss, but even release several cytokines/chemokines beneficial to tissue regeneration and repair.

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Abbreviations: CEA, cultured epithelial autografts; DPF, designated pathogen free; GM-CSF, granulocyte-macrophage colony stimulating factor; IC, immunocytochemistry; IL, interleukin; PERV, porcine endogenous retrovirus; TGF, tumour growth factor; TNF, tumour necrosis factor; WB, Western immunoblotting; NPSS, neonatal pigs split-skin

Key words: cultured epithelial autografts, neonatal pigskin, organ culture, xenografts, biological dressings, IL-1α, IL-6, GM-CSF, TNF-α, TGF-ß

Introduction

Extensive areas of human skin loss, e.g. burn wounds of 6,000-19,000 cm^2 or more, are often difficult to cover with a suitable dressing. A burn must be closed swiftly and completely with a temporary, skin-like dressing (for an optimum result), to improve its clinical course (1). Autografts are the ideal cover for full-thickness burns, being easily re-vascularised and thus 'taking' with a high success rate without inducing any rejection reaction (1). Unfortunately, the size of available donor sites is inversely proportional to the extent of burn wounds (2), and technical refinements, such as repeated harvesting of donor sites and meshed autograft expansion, only partly solve the problem, while bringing in the dangers of local desiccation and infection (3). On the other hand, cultured epithelial autografts (CEAs) have given unpredictable and inconsistent clinical results because of low 'take' rates (15-21%) due to non-optimal wound bed conditions (4), and second-set rejection responses evoked by the persistence of foreign (human or mouse) fibroblasts used as feeder layers (5). Allografts (or homografts) from living or dead donors are used by themselves or in association with autografts to cover extensive wounds (6). Allografts elicit earlier epithelisation and better end-results, but donor availability is limited by cultural and religious factors and by ongoing diseases such as tumours and infections. Repeated allograft applications trigger sensitisation and accelerated rejection of the second- or third-set grafts, unless immunosuppressive therapy is used (6,7). Moreover, allografting entails the risk of transmitting infectious agents to the host (8). The use of cultured allo-epithelial grafts is hampered by the same restraints mentioned for allografts and CEAs (5). The exploit of using xenografts in burn care was pioneered by Reverdin in the 19th century (9). Thenceforth, skin flaps taken from a number of animal species, such as the shark, frog, chicken, pigeon, rat, sheep, goat, cat, dog, and cow, have been used as biological dressings for sizeable skin wounds in animals and humans (10-15). Chemically-treated bovine skin (Dermodress®) has also been applied as a long-term skin substitute onto deep

burn wounds (16). However, just as happens for allografts (5), permanent transplantation of animal skin is precluded by immunologic rejection (17). During the past 40 years, adult pig skin in various forms (i.e. fresh, freeze-dried, pre-irradiated frozen, incorporating neomycin and/or silver ions, formaldehyde-fixed, glycerinated, lyophilised or non-lyophilised glutaraldehyde-fixed, and chlorhexidine-treated) has been by far the most popular xenograft used to cover burn wounds, donor sites, meshed autografts, areas of partial thickness skin loss, and non-healing cutaneous ulcers of various aetiology (18-29). Adult porcine skin apparently has all the features of an ideal wound protective cover, such as tensile strength; tissue adherence; flexibility; water-vapour permeability; heat retention; a human skin-like histological structure (18) unless deeply altered by chemical treatments; anti-bacterial and pain relieving effects; lack of antigenicity; no toxicity; low cost; and long shelf-life (18-29). Moreover, adult porcine skin xenografts were never vascularised (15,18,20,30,31), even though being at times partly included within the healing tissue (32). Despite a slight cellular infiltration at the wound bed/xenograft boundary, no significant levels of anti-donor pig antibodies were induced and no immunological reaction or rejection evoked (15,33). The pig skin dressing desiccated and detached from the wound bed, thereby acting more as a collagen prosthesis than as a true xenograft (34). However, the above mentioned advantages of the various forms of adult pig skin dressing were negated by several studies documenting that the removal of neomycin after reports of toxic and untoward reactions rendered porcine dressings ineffective; that there was no difference in the beneficial effects on wound healing observed between adult pig skin applied with mafenide acetate (Sulfamylon®) cream and mafenide alone; that lyophilised porcine skin was not found to be more beneficial than petrolatum gauze or coarse mesh gauze changed thrice every day; that formalin-fixed pig skin behaved by far as the worst dressing; and finally, that adult pig skin did not confer any advantage over or protect any better than simple gauze (35-38). Hence, during the last two decades the use of adult pig skin has been declining steadily in clinical settings, being at present restricted to a few centres (39).

In this study, we examined the feasibility of using neonatal porcine split-skin (NPSS) strips, a novel kind of wound dressing. The results showed that frozen/thawed NPSS strips retain their viability, metabolism, and capacity for producing various cytokines/chemokines that play crucial roles in skin pathophysiology just as fresh NPSS strips do. Moreover, our findings also demonstrated that, once grafted onto severe burn wounds *in vivo*, frozen/thawed NPSS strips produced, for at least one week, several cytokines/chemokines, thereby acting both as a cover and as a biological dressing favouring the healing process.

Materials and methods

NPSS strip preparation and handling. All procedures employed for animal husbandry conditions, disease surveillance and screening procedures, and tissue banking standards were in keeping with Food and Drug Administration (FDA) guidelines (40) and the World Health Organisation (WHO) (41). Neonatal piglets were from sows raised in a barrier facility and maintained free of designated pathogens (Designated Pathogen Free or DPF animals) via *ad hoc* surveillance programs and veterinary care. Piglets were directly washed in a warm antiseptic bath, anaesthetised with ethyl ether, and sacrificed by an intra-cardiac injection of KCl solution. Stripped hides were dipped into an antiseptic solution, stripped of hair, and put into RPMI-1640 medium (Sigma Italia S.p.A., Milan). After cleansing of subcutaneous fat lobules, the hides were cut into split-thickness (1.5-mm-thick) strips (width, 7.5 cm; mean length, 10-20 cm) using a dermatome (Nouvag AG, Goldach, Switzerland). Next, NPSS strips were incubated for 2 h in RPMI-1640 medium fortified with antibiotics and zidovudine (42). They were transferred to a 20% glycerol-80% RPMI-1640 medium and put into three sterile plastic envelopes that were heat-sealed and quarantined in a dedicated deep freezer. In parallel, samples taken from each piglet hide, NPSS strip and their respective soaking media underwent microbiological, mycological, and virological analyses. NPSS strips found to be sterile (average, 98%) were transferred to an *ad hoc* deep freezer to be stored for up to 6 months. When needed, sterile frozen NPSS strips were taken out of this repository, rapidly thawed at 45˚C (henceforth they will be referred to as frozen/thawed strips), and washed with fresh RPMI-1640 medium to be either set into organ cultures *in vitro* or enclosed in three sterile heat-sealed envelopes sent by courier to the Burn Wound Care Unit (Major Hospital, Verona) to be grafted, as an ultimate supportive measure, onto patients suffering from severe burns. In five different instances, punch biopsies (4 mm in diameter) were taken from the frozen/thawed NPSS strips prior to (0-time), and 3 and 7 days after grafting *in vivo* to assess tissue production of various porcine cytokines/chemokines.

In vitro organ cultures of NPSS strips. Both freshly prepared and frozen/thawed NPSS strips were cut into 2.5x2.5 cm pieces that were cultured at 37˚C inside the square wells of Falcon flasks in the presence of 95% air/5% CO_2. Such organ cultures were kept for up to three weeks. The growth medium [RPMI medium fortified with antibiotics and 5% (vol/vol) adult horse serum (Cambrex Bio Science, Milan)] was changed with a fresh one every fourth day. Cell-conditioned media were stored at -80˚C to be subsequently analysed by biochemical means.

Metabolic assays of cultured NPSS strips. The uptake of L-glutamine was evaluated as follows. Twenty microliters of each NPSS strip-conditioned growth medium sample was incubated for 15 min at 37˚C with 0.1 M sodium acetate, pH 4.9, in the presence of 0.05 units of glutaminase (EC 3.5.1.2) to convert the L-glutamine into glutamate and ammonium (NH_3). The resulting amount of ammonium was next determined by the reductive amination of 3.4 mM 2-oxo-glutarate, using 12 units of L-glutamate dehydrogenase (GLDH) and 0.23 mM reduced NADPH in 0.1 M sodium phosphate, pH 7.4. Finally, the decrease in absorbance at 340 nm, due to the oxidation of NADPH, was quantified, and this was proportional to the concentration of the L-glutamine remaining in the cell-conditioned medium samples. The

utilisation of D-glucose was appraised in the NPSS strip-conditioned growth medium samples by a glucose oxidase assay according to Trinder *et al* (43) (Sigma). The lactic acid released into the NPSS strip-conditioned growth medium samples was assessed via a lactate oxidase assay according to Barham *et al* (44), again as developed by Sigma. The urea secreted into the NPSS strip-conditioned growth medium samples was assessed via a specific commercial kit according to the manufacturer's instructions (Sigma). In each of the above instances as well as in the case of the secretion of various cytokines/chemokines (see below), cumulative curves were constructed from the data pertaining to fresh or frozen/thawed NPSS strips. Each experiment was performed at least five times.

ELISA assays of porcine cytokines/chemokines released by cultured NPSS strips. Growth medium samples conditioned by contact with either freshly prepared or frozen/thawed NPSS strips were assayed for a set of porcine cytokines/chemokines by using antibodies not cross-reacting with the corresponding human cytokines. A commercial ELISA kit was used to assay IL-6 (R&D Systems Inc., Minneapolis, MN). Moreover, we used various ELISA tests developed in our laboratory to assay IL-1α, GM-CSF, TGF-ß, and TNF-α. For each ELISA a 96-well plate was coated with 50 μl of growth medium diluted 1:1 in 0.1 M carbonate buffer pH 9.0, overnight at 4˚C, and then blocked with 200 μl of PBS/1% bovine serum albumin (BSA) for 1 h at room temperature. After washing with PBS/0.05% Tween solution, a polyclonal goat or rabbit antibody specific for porcine IL-1α, GM-CSF, TGF-ß, and TNF-α (R&D Systems Inc.) was added at a concentration of 1 μg/ml in PBS/5% BSA and incubated for 1 h at room temperature. After removal of excess antibody, an appropriate second antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1000 in PBS/5% BSA was added. Following a wash procedure to remove any unbound antibody, the chromogen substrate tetramethylbenzidine (TMB) solution was added to the wells and the enzymatic colorimetric reaction was stopped by adding 100 μl of 0.1 M H_2SO_4 to each well. The absorbance was read at 450 nm having blanked the plate reader against a chromogen blank composed of 100 μl each of TMB solution and stop solution. For each ELISA a standard curve was generated using recombinant porcine cytokines/chemokines (R&D Systems Inc.). The detection limits of these assays were: IL-1α, 0.16 ng/well; GM-CSF, 0.2 ng/well; TGF-ß, 1.6 ng/well; TNF-α, 0.5 ng/well; and IL-6, 10 pg/ml. The results were expressed as secretion values per triplicate wells per 8-10 distinct experiments.

Histology and immunocytochemistry (IC). Samples of freshly prepared or frozen/thawed NPSS strips and biopsies of frozen/thawed NPSS strips taken just prior to grafting (experimental 0-time) *in vivo* or 3 and 7 days after grafting were instantly frozen using precooled (-80˚C) isopentane in liquid nitrogen (45). Next, sections were cut at 5-to 15-μm thicknesses in a 1720 Digital cryostat (Leitz Lauda, Milan, Italy), mounted on aminopropyl-triethoxysilane (APES)-coated slides, fixed for 20 min in acetone at -20˚C, and dried (45). Tissue sections were subsequently stained with Mayer's haematoxylin and eosin (H&E) (46). The procedures followed for IC were those described by Jackson and Blythe (45). Tissue sections were washed twice with PBS containing BSA (1.0 % w/v) and NaN_3 (0.1% w/v), and incubated for 60 min at room temperature with primary antibodies (at 1.0 μg ml^{-1}) specifically directed against porcine (no human cross-reaction) IL-1α, IL-6, GM-CSF, and TNF-α (R&D Systems Inc.). Next, tissue sections were washed thrice with PBS-BSA (1%) solution and incubated for an additional 1 h at room temperature with proper second antibodies (1:100 dilution) conjugated with alkaline phosphatase (all from Santa Cruz Biotechnology). Specific IC colours were developed with Fast TR naphtol or Fast Red AS-MX tablet sets (both from Sigma). Control tissue sections were run in parallel, the primary or secondary antibody being omitted from the procedure. Specimens were examined under an Olympus BX-60 microscope and photographed with an Olympus 3300™ digital camera.

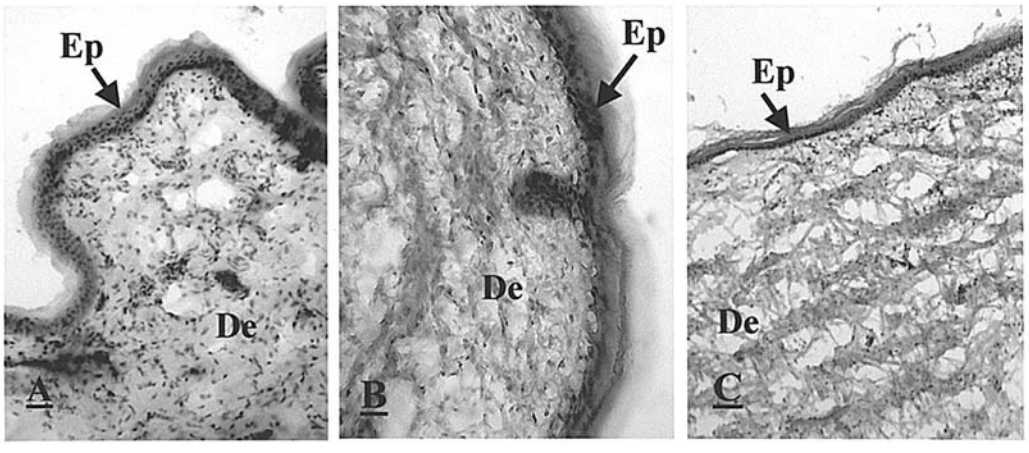

Figure 1. The histological structure of processed NPSS strips as seen in 5- to 8-μm-thick cryostat sections. For technical details, see the Materials and methods section. (A) Freshly prepared NPSS strip. (B) Frozen/thawed NPSS strip; note the well-preserved structure of the tissue compared to the fresh tissue shown in A. (C) A sample of frozen (for three months at -80˚C)/thawed NPSS strip set into an *in vitro* organ culture for 16 days. The epidermal cell layers are still distinct and a visible stratum corneum indicates that the epidermis is undergoing normal differentiation/apoptosis. The structure of the cultured dermis is similar to that of specimens A and B save for a minor degree of oedema. H&E staining. Magnification, x40. Ep, epidermis; De, dermis.

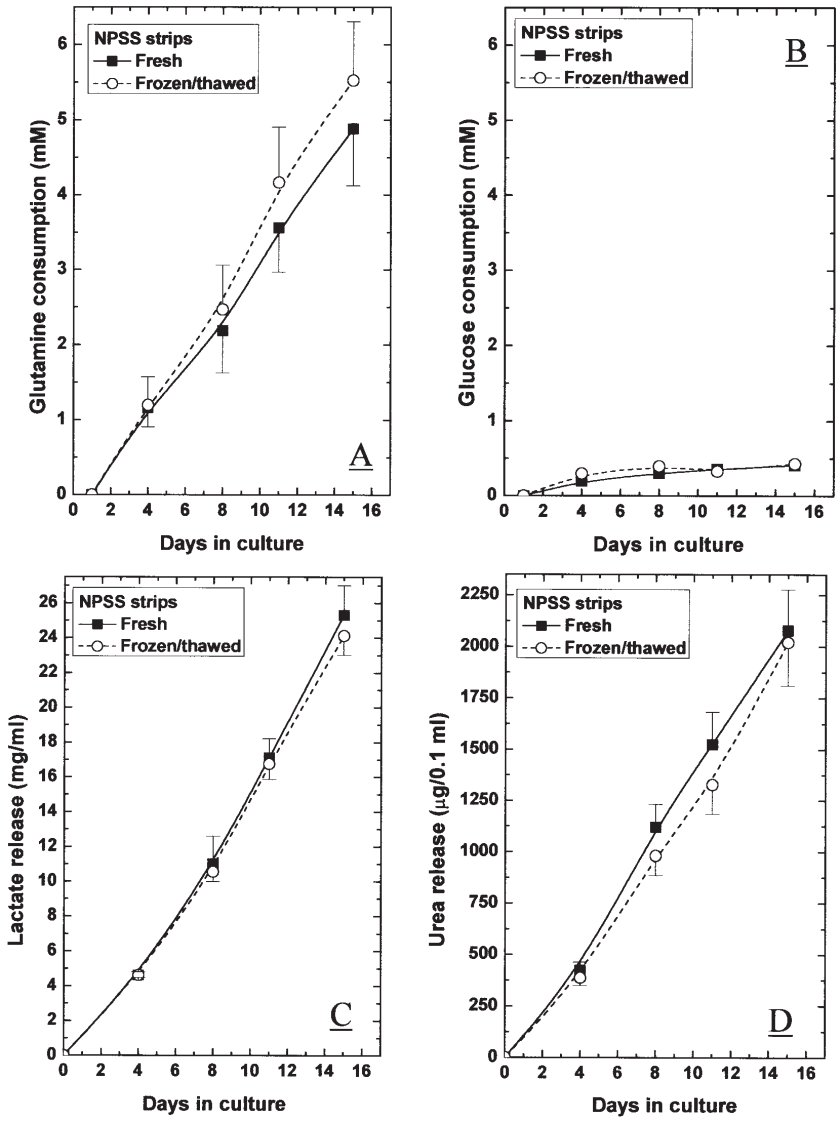

Figure 2. Energy-linked metabolic processes similarly occur in fresh and in frozen/thawed NPSS strips set into organ cultures *in vitro*. (A) The similar consumption of L-glutamine by fresh and by frozen/thawed NPSS strips shows that this amino acid is, in both instances, as *in vivo*, the main energy-producing fuel (49). (B) D-glucose is only marginally taken-up by fresh and by frozen/thawed NPSS strips. (C) The comparable release of lactate into the RPMI-1640 medium by fresh and by frozen/thawed NPSS strips. In the skin *in vivo*, lactate is a by-product of L-glutamine and D-glucose metabolisms (49). (D) The release of urea into the RPMI-1640 medium by fresh and by frozen/thawed NPSS strips is quite similar, revealing an ongoing turnover of amino acids and proteins. Points on the curves are the means ± SEM of triplicate wells from 8-10 distinct experiments for each group of samples. No significant statistical difference was obtained between the mean values of time-corresponding samples.

Western immunoblotting (WB). Extraction of total proteins from punch biopsies (diameter, 4 mm) of frozen/thawed NPSS strips was carried out just prior to grafting *in vivo* (experimental 0-time) and 3 and 7 days later. The skin samples were washed with cold PBS and homogenised in T-PER™ tissue protein extraction reagent (Pierce Chemical Co., Rockford, IL) containing a complete EDTA-free protease inhibitor cocktail (Roche Diagnostics S.p.A., Monza, Italy). Subsequent procedures for WB were carried out according to Chiarini *et al* (47). The protein contents of the samples were assayed by Bradford's method (48) using bovine serum albumin as a standard. Equal amounts (25-30 μg) of proteins from each sample were boiled in sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% w/v SDS, 5% w/v ß-mercaptoethanol, 10% v/v glycerol, and 0.002% w/v bromphenol blue) and electrophoresed in 12% w/v SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane (0.45 μm; Pall Life Sciences, Ann Arbor, MI). To immunodetect the porcine cytokines/chemokines IL-1α, IL-6, GM-CSF, TNF-α and TGF-ß, the blots were probed with specific goat or rabbit IgG polyclonal antibodies used at a final dilution of 1.0 μg ml^{-1}; (all from R&D Systems Inc. except the anti-IL-6 antibody from BioSource International Inc., Camarillo, CA). Blots were next incubated with alkaline phosphatase-conjugated anti-goat or anti-rabbit IgGs (Santa Cruz Biotechnology, Inc.), and stained with BCTP/NBT liquid substrate reagent (Sigma). Developed blots were scanned in an Odyssey® Infrared imaging system (Li-Cor Biosciences Inc., Lincoln, NE) and the integrated intensity of each specific protein band was accurately quantified using proprietary software.

Statistical analysis. The Student's t-test was used to compare mean values and a significance level of 0.05 was chosen.

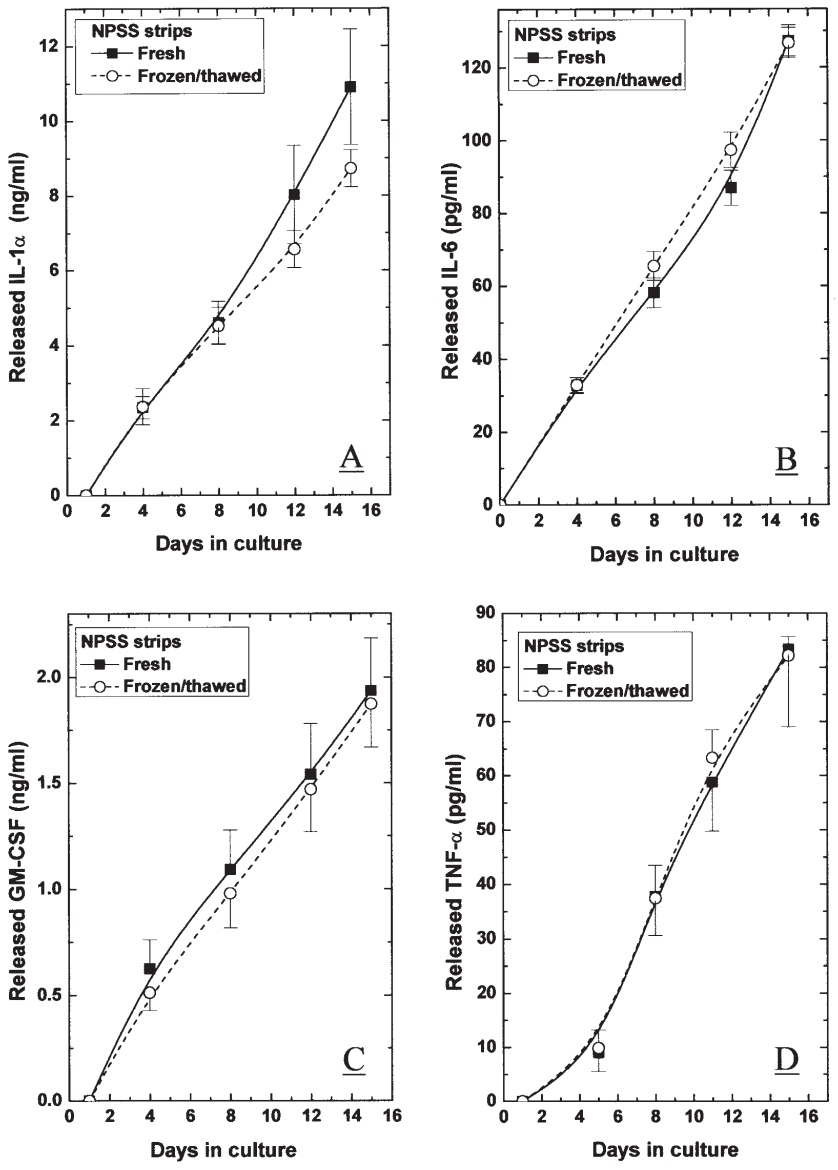

Figure 3. Secretion of the various porcine cytokines/chemokines into the RPMI-1640 medium is similarly upheld by fresh and by frozen/thawed NPSS strips set into organ cultures *in vitro*. Similar amounts of IL-1α (A), IL-6 (B), GM-CSF (C), and TNF-α (D) were released with time by the two groups of specimens. The assay procedures employed are detailed in the Materials and methods section. Points on the curves are the means ± SEM of triplicate wells from 8-10 distinct experiments for each group of samples. No significant statistical difference was obtained between the mean values of time-corresponding samples.

Results

NPSS strips can be prepared in bulk amounts (e.g. 1.5-2.0 m^2 from 15-20 piglets) by two workers in the course of a single day. Our first endeavour was to establish whether the laboratory procedures required to produce frozen/thawed NPSS strips would significantly alter their biological characteristics (e.g. histology, energy metabolism, and cytokine/chemokine release) compared to those exhibited by fresh NPSS strips.

Observations carried out under a light microscope of H&E-stained cryostat sections of frozen/thawed NPSS strips revealed a well preserved histological structure, which compared with those of the fresh strips (Fig. 1A and B).

Moreover, analysis of cryostat sections of frozen/thawed NPSS strips revealed that, after two-three weeks of staying in organ cultures *in vitro*, the epidermal layers were still well preserved and were undergoing the expected differentiation/apoptosis (Fig. 1C), while the dermal connective tissue was slightly oedematous (Fig. 1A and C).

These histological findings were in keeping with the results of biochemical assays showing that frozen/thawed NPSS strips could, in the course of 15 days in organ culture, both maintain a skin-specific energy metabolism and secrete various cytokines/chemokines at levels not significantly different from those exhibited by fresh strips directly set into culture. As shown in Fig. 2, cultured, both fresh and frozen/thawed NPSS strips similarly elected L-glutamine (Fig. 2A) as their principal energy supply instead of D-glucose (a typical skin metabolic feature) (Fig. 2B) (49) and continued releasing like amounts of two catabolites, lactic acid and urea (Fig. 2C and D, respectively) into the growth medium.

To note, once set into organ cultures *in vitro*, both freshly prepared and frozen/thawed NPSS strips continuously

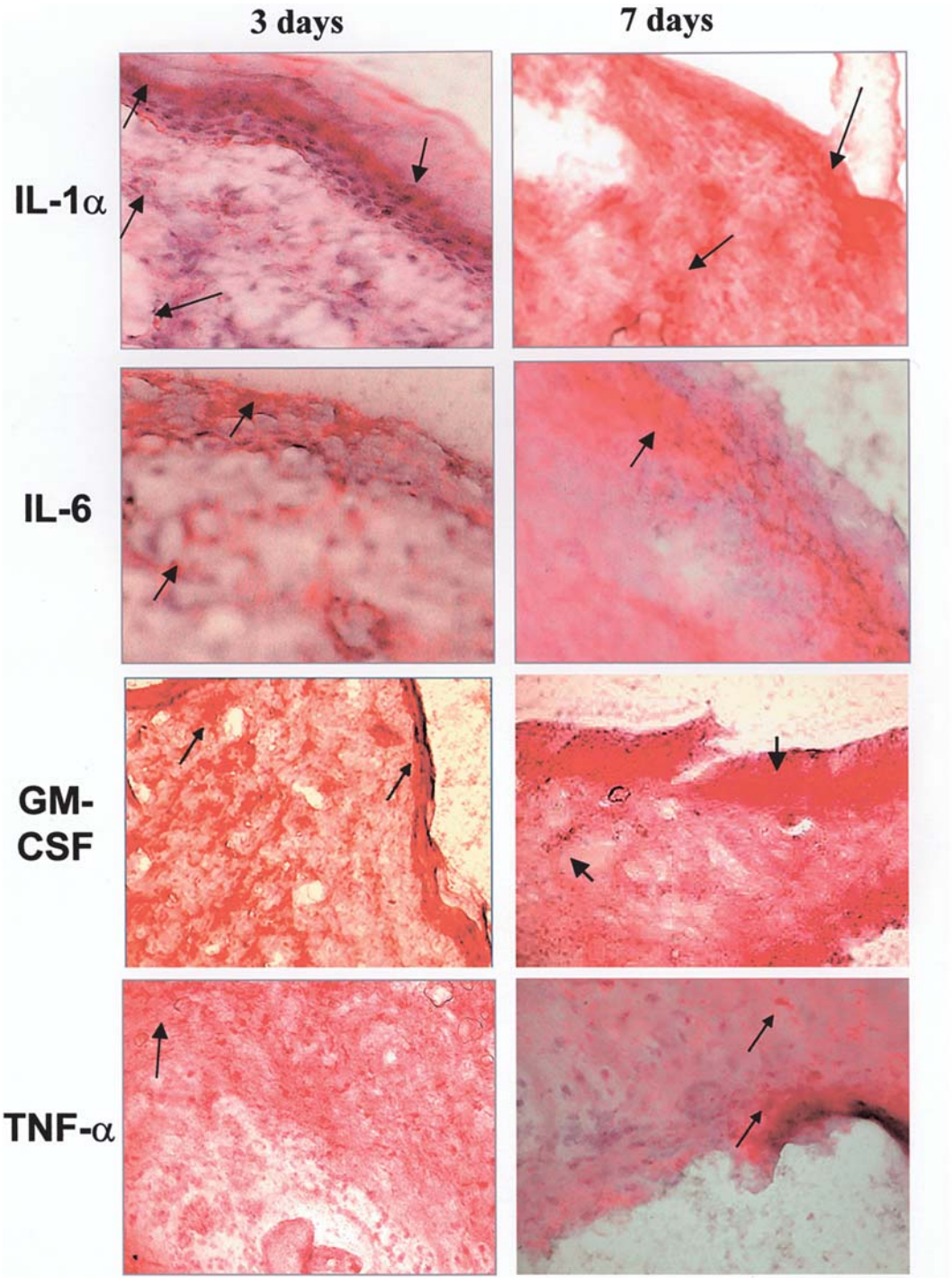

Figure 4. Frozen/thawed NPSS strips produce immunocytochemically detectable amounts of various porcine cytokines/chemokines after 3 and 7 days of grafting onto deep burn wounds *in vivo*. Note the increased intensity of the antibody decoration (in pink and red; arrows) in 7-day samples vs 3-day specimens for each cytokine/chemokine examined. The specific primary antibodies used did not cross-react with the corresponding cytokines of human origin. A light haematoxylin counterstaining reveals that the histological structure of the grafted NPSS strips is still well-preserved even after 7 days of grafting *in vivo*. Original magnification, x40 or x100.

secreted reciprocally matching amounts of four porcine cytokines/chemokines, IL-1α, IL-6, GM-CSF, and TNF-α, all of which are known to play relevant roles in skin physiopathology (50) (Fig. 3). Conversely, TGF-ß was undetected in the sampled conditioned growth media (not shown).

Collectively, these findings showed that frozen/thawed NPSS strips did not differ from fresh NPSS strips in histological structure, energy metabolism, and the ability to release assayable amounts of the cytokines/chemokines.

The second aim of the present study was to establish whether, once grafted onto deep burn wounds *in vivo*, frozen/thawed NPSS strips would remain biologically active for a long period of time or rapidly become inert dressings. The results of the immunocytochemical and Western blot analyses support the former alternative. Notably, at 0-time (i.e. just prior to grafting) only porcine IL-6 was expressed by the frozen/thawed NPSS strips (Fig. 5B). However, three days after grafting *in vivo*, porcine IL-1α, IL-6, GM-CSF, and TNF-α were distinctly expressed by the deeper epidermal

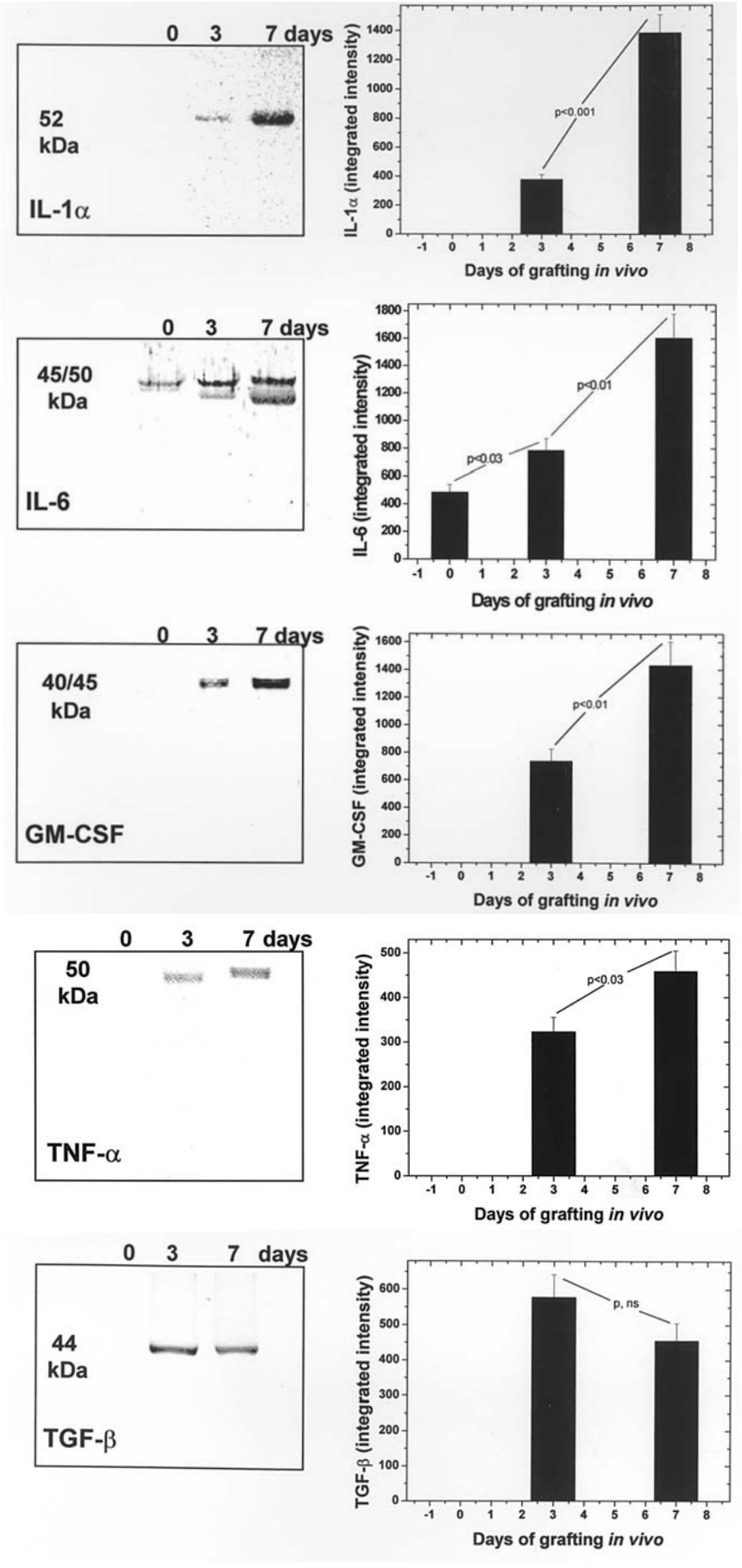

Figure 5. (Left panels) WB analysis of total protein extracts from biopsy specimens shows that frozen/thawed NPSS strips still produce various porcine cytokines/chemokines after 3 and 7 days of grafting onto deep burn wounds *in vivo*. The immunoblots shown are typical for each cytokine investigated. (Right panels) Densitometric evaluation of the specific protein bands for each cytokine. Bars are ± SEM of five distinct biopsies. The specific primary antibodies used for WB did not cross-react with the corresponding cytokine of human origin.

layers and by sparse dermal cells (Fig. 4). In addition, 7 days after grafting *in vivo*, the expression of the same four porcine cytokines/chemokines became increasingly more intense in both the epidermal and dermal layers (Fig. 4). Light counterstaining with haematoxylin of the IC specimens showed that the histological structure of the frozen/thawed NPSS strip biopsies was well-preserved at day 3 and 7 of grafting *in vivo* (Fig. 4).

Analysis by WB of the total protein extracts of the biopsies from the frozen/thawed NPSS strips grafted onto burn wounds *in vivo* substantiated the immunocytochemical findings (Fig. 5). Only porcine IL-6 was expressed by the frozen/thawed NPSS strips prior to grafting (at 0-time) (Fig. 5B). Frozen/thawed NPSS strips at grafting day 3 produced discrete amounts of IL-1α, IL-6, GM-CSF, and TNF-α. Densitometric analysis of the specific protein bands showed that the expression of these cytokines was significantly more intense by day 7 after grafting *in vivo*: IL-1α, +275%, $p<0.001$; IL-6, +104%, $p<0.01$; GM-CSF, +100%, $p<0.01$; and TNF-α, +42%, $p<0.03$ (Fig. 5). IL-6 expression was also greater at day 3 vs day 0 (+66%, $p<0.03$). On the other hand, the expression of TGF-ß was absent at 0-time, but detectable by day 3 and not significantly changed by day 7 of grafting *in vivo* (day 7 vs day 3, -21%, p not significant) (Fig. 5). Therefore, frozen/thawed NPSS strips can resume and even increase or maintain the production of certain cytokines/chemokines for at least 7 days after being grafted onto burn wounds *in vivo*.

Discussion

The present findings show that frozen/thawed NPSS strips maintain their viability for long periods, as attested by their normal histological structure and their specific metabolic capabilities, both in organ cultures *in vitro* and after grafting onto burn wound beds *in vivo*. Consequently, they have all the properties needed to perform both as ideal covering bandages (1,2) and as biologically active wound dressings. The latter capability is not shared by the various chemically- or radiation-treated, hence biologically inert adult pig skin dressings commercially available and clinically used both in the past and at present (18-29).

In fact, when temporarily grafted *in vivo*, frozen/thawed NPSS strips not only close the burn wound, thereby preventing the loss of significant amounts of water, electrolytes, and precious proteins, blocking the penetration of foreign infectious micro-organisms, and reducing pain (and the need for analgesic medications), but even favour the regenerative and healing processes via the production and extracellular release of a complex set of beneficial cytokines/chemokines (50). The preliminary clinical results of grafting frozen/thawed NPSS strips onto severely burnt patients have consistently confirmed the favourable effects of such biologically active dressings, used by themselves or in combination with underlying meshed skin autografts, on wound healing in the complete absence of any adverse side effects and of any rejection or inflammatory reactions (manuscript in preparation).

An important advantage from the practical standpoint is that frozen/thawed NPSS strips can be prepared in large amounts, checked for sterility, stored in a deep freezer, and when required promptly made ready for grafting *in vivo*. This means that, at short notice, even an adult patient with 95% burns, a surface of 1.7 m^2 or more, can be covered with frozen/thawed NPSS strips. Moreover, when used alone or in conjunction with meshed skin autografts or allografts, these same strips may benefit even split-skin graft donor sites (11,20), chronic skin ulcers of any aetiology, and other pathological skin conditions such as toxic epidermal necrolysis (51).

Here, it must also be stressed that, at variance with allografts, especially those from cadavers, frozen/thawed NPSS strips are endowed with highly reproducible long-term biological properties both in *in vitro* cultures and, most important, after grafting *in vivo*. Conceivably, the wound bed fluid infiltrates the grafted NPSS strips maintaining their hydration and providing nutrients for at least a week before desiccation, avascular necrosis, and spontaneous detachment from the wound bed ensue. Thus, the grafted frozen/thawed NPSS strips not only continue preferring L-glutamine as the main energy-producing fuel, a specific feature of skin (49), but even continue synthesizing and secreting cytokines/chemokines that play key roles in skin pathophysiology (50). Reportedly, IL-1α is importantly involved in inflammatory skin conditions and in epidermal cell growth and repair processes via members of the IL-1R (receptor) family (52,53). IL-6 is a multifunctional cytokine of the haemopoietin family comprising amongst others the granulocyte-colony stimulating factor (G-CSF) and acting via the gp130 signal-transducing subunit (54). IL-6 operates as a growth factor for keratinocytes either via its specific receptor signalling (55) and/or by inducing the production and release of keratinocyte growth factor (KGF) by dermal fibroblasts (56). By means of paracrine and autocrine receptor-mediated loops, IL-6 also may enhance the proliferation of human adult dermal fibroblasts and/or up-regulate the production of collagen, glycosaminoglycans (GAGs), interstitial collagenase, and stromelysin-1 by such cells, thereby favouring the inflammatory processes related to wound healing (57). On its own part, the haemopoietin GM-CSF (54) is a pleiotropic cytokine secreted by keratinocytes, fibroblasts, endothelial cells, dendritic cells, macrophages, and lymphocytes; cell types involved in wound healing. GM-CSF plays complex roles like accelerating re-epithelisation, enhancing neovascularisation, increasing the formation of granulation tissue, and inducing secondary cytokines such as IL-6, TGF-ß, and IFN-γ (58). Via its ubiquitous receptors, TNF-α is involved in various relevant aspects of skin wound healing such as cell proliferation, cell differentiation, inflammation, and immunomodulation (59, and refs. therein). Finally, the members of the TGF-ß family are pleiotropic cytokines that i) orchestrate a large number of cellular activities related to wound healing and tissue fibrosis via specific receptors and Smad-protein-regulated intracellular signalling cascades in keratinocytes, fibroblasts, endothelial cells and monocytes, and ii) modulate dermal-epidermal interactions in wound repair (60, and refs. therein). To note, the pro-fibrotic activity of TGF-ß which is mediated via the induction of connective tissue growth factor (CTGF) expression in fibroblasts, is counteracted by TNF-α (61). Therefore, just as

in the case of whole skin or cultured epithelial autografts and allografts, frozen/thawed NPSS grafts i*n vivo* may work not only as mechanical bandages, but even as pharmacologic tools providing growth factors and cytokines/chemokines that accelerate regeneration/repair processes (62). Examples of human cells responding to porcine cytokines have been reported (63).

It should be recalled here that using living human skin allografts entails the risk of transmitting infectious agents to patients, particularly viruses such as the Epstein-Barr virus, cytomegalovirus, HIV, Kaposi-sarcoma-associated herpes virus and hepatitis viruses (8,63-66), as well as prions (67). Such risks are attenuated, but not entirely removed, by procedures that also do away with allograft viability (68). On the other hand, transmission of xenozoonoses (xenoses) by grafting living pig tissues or organs is partly restricted by using DPF animals undergoing adequate surveillance programs and veterinary care (69). The infectivity of porcine viruses, especially endogenous retroviruses (PERVs), for humans *in vivo* is still an object of controversy. However, it must be stressed that all published retrospective studies on human patients or subjects exposed to living pig tissues (e.g. following the grafting of pig islet cells or fetal neurons, or vascular connections to extra-corporeal pig livers or kidneys, or such as butchers who are in constant contact with fresh pig tissues and blood) have failed to demonstrate the presence of PERV DNA or RNA sequences or the occurrence of a PERV seroconversion in the hosts or exposed workers (71-85). Conversely, it has been proven that natural antibodies prevent the transmission of PERV to human cells *in vivo* (81). Furthermore, no evidence exists that PERVs infect other mammalian species, including nonhuman primates (80,84). Nevertheless, adequate caution, like the exposure of NPSS strips to zidovudine (42), and patient follow-up are in order for the temporary grafting of frozen/thawed NPSS strips onto wounds *in vivo*. Additional procedures, such as the inhibition of PERV expression by RNA interference or the remodelling of the PERV envelope glycoprotein (86-88), will further ensure the safety of using frozen/thawed NPSS strips in clinical settings.

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