Radiosensitivity of Angiogenic and Mitogenic Factors in Human Amniotic Membrane

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Amnionic membrane as a temporary biological dressing remains as a beneficial and cost-effective means of treating burns in developing countries. This medical application is attributed mainly to placental structural and biochemical features that are important for maintaining proper embryonic development. Since fresh amnions are nevertheless for straightforward clinical use and for preservation, radiation-sterilization is been performed to improve the safety of this placental material. However, like any other sterilization method, gamma-radiation may induce physical and chemical changes that may influence the biological property of the material. Thus, the aim of this study is to compare the effects of various levels of radiation-sterilization protocols for human amnions on angiogenic (neovascularization) and epithelial-mitogenic activities, both of which are physiological processes fundamental to wound healing.

Water-soluble extract of non-irradiated amnions demonstrates a strong stimulatory effect on both cell proliferation and angiogenesis. No change in biological activity is seen in amnions irradiated at 25 kGy, the sterilization dose used by the Philippine Nuclear Research Institute (PNRI) for the production of radiation-sterilized human amniotic membranes (RSHAM). However, it appears that amniotic angiogenic factors are more radiosensitive than its mitogenic components, evident from the depressed vascularization of the chorioallantoic membrane (CAM) exposed to 35 kGy-irradiated amnions. The dose of 35 kGy is at present the medical sterilization dose used at the Central Tissue Bank in Warsaw (Poland) for the preparation of their amnion allografts.

The use of human amniotic membranes for wound coverage was first published 1913 (Sabella, 1913). Until now, outcomes of clinical trials on its application in supportive care for wound and burn cases, dermabrasions and skin ulcers, plastic surgery, laryngology, and for spinal and ocular surgical procedures are still being reported (Dua & Azuara-Blanco, 1999). Human amnion is a readily available biological dressing material that not only prevents the discharge of plasma from burn wounds, but also alleviates pain and prevents sepsis. Its is a preferred wound coverage because the amniotic membrane stimulates re-epithelialization and wound granulation; reduces loss of fluid, protein, heat and energy; increases mobility of the injured area; and served as an ideal wound cover next to the patient’s own skin (Sharma et al., 1985).

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In developing countries, the use of human amnions is cited as the most cost-effective strategy in the management of burn wounds considering the supply of the material, efficacy and patient acceptability (Ramakrishnan & Jayaraman, 1997). Dino et al. (1966) pioneered the tissue banking of amnions in the Philippines. However, his group reported practical difficulties in preserving and applying fresh membranes in a hospital setting. The first demonstration of the advantages of radiation processing for the preparation of human amnion dressings was made possible through the PNRI-PGH collaboration and the establishment of a Tissue Bank at the Philippine General Hospital (Agcaoili et al., 1988). Since then RSHAM has been manufactured by PNRI for distribution among burn patients within Metro Manila.

The process of wound healing involves inflammation, cellular proliferation, tissue remodeling, re-epithelization, granulation and finally, wound contraction. Two key factors shape the design and acceptability of biological wound coverage: the ability to induce cellular proliferation to promote epithelization-remodeling at the damaged site and the capacity to recruit new capillaries by angiogenesis for efficient provision of oxygen and nutrients to proliferating cells. In fact, the presence as well as the combination of growth factors to control appropriate healing is underscored by many findings. There are five major growth factors that contribute significantly to the wound healing process: epidermal growth factor (EGF), transforming growth factor-b (TGF-b), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF) and the fibroblast growth factor (FGF) family. Interestingly, amnion membrane also possesses a number of important cytokines, e.g. platelet-activating factor (PAF), basic FGF, hepatocyte growth factor (HGF), placental prolferin, TGF-b and EGF (Gospodarwicz, Neufeld & Schweigerer, 1986; Zhu, Word & Johnston, 1992; Hansbrough, 1992; Jackson et al., 1994). Autocrine functioning interleukins (ILs) have been ascribed new roles in wound healing, i.e. the induction chemotactic responses of keratinocytes (Michel et al., 1992), the up-regulation of integrin expression (Reusch et al., 1990) and stimulation of angiogenesis (Hu, Hori and Fan, 1993). Amnions also appear to express these pro-inflammatory cytokines (IL-1, IL-6, IL-8 and tumor necrosis factor (TNF)-a that activate local protective response in the presence of invading bacteria as well as their endotoxins (Trautman et al., 1992; Mitchell et al., 1991; Dudley et al., 1993). Amnions also appear to express these pro-inflammatory cytokines (IL-1, IL-6, IL-8 and tumor necrosis factor (TNF)-a that activate local protective response in the presence of invading bacteria as well as their endotoxins (Trautman et al., 1992; Mitchell et al., 1991; Dudley et al., 1993). Based on these facts, several types of biologic dressings and temporary skin replacements containing many of these growth factors and cytokines have been developed to enhance healing yet their applications in human chronic wounds frequently failed to achieve success. This indicates that some wounds require a balanced proportion and an orderly progression of specific growth factors during healing and such may not be remedied by simple application of a growth peptide to a synthetic dressing (Falanga, 1993). Therefore, it is obvious that there is still a need for continued tissue banking activities of amnion dressings not only in the Philippines but also in many parts of the world (Tyszkielewicz et al., 1999, Gamero & Perez, 1998; Djefall et al., 1998). Despite the recent developments in tissue biotechnology wherein new biomaterials are being synthesized, none could perfectly mimic the function and physiology of a regenerating skin.

This study was designed to directly analyze the effects of various levels of absorbed radiation dose used during the preparation of human amnion dressings. We examined the differences in the stimulating growth of epithelial cells in vitro and in recruiting capillaries on the CAM to understand the fundamental determinants of wound healing, that is epithelial mitogenicity and angiogenesis (neovascularization).

**Materials and Methods**

**Sample Collection and Preparation.** Amnion membrane was obtained from 155 mothers seronegative for hepatitis B and HIV from the Obstetric Department of the East Avenue General Hospital from June 15-30 2001. HIV testing was performed at the Research Institute for Tropical Medicine using agglutination tests. Hepatitis B screening was done at the PNRI using radioimmunoassay (RIA) for Hep B Antigen.

Tissues with meconium stain were discarded. Each healthy placenta was washed with tap water to remove blood. The amnion membrane was excised, washed with 5% NaH2ClO4 and lyophilized to 15% moisture level prior to irradiation. Moisture content was monitored by gravimetric method. The dried membranes were packaged in polyethylene bags and irradiated at 15, 25 and 35 kGy in a Co-60 source (AECL Gamma Cell) at the PNRI (AAMI, 1991; ISO, 1993). Water-soluble fractions of the irradiated amnion membranes were freeze-dried and stock solutions of the extracts were prepared in phosphate buffered saline pH 7.2 (PBS). After filter-sterilization through a 0.2 um pore, the extracts were stored at –70°C until used for cell proliferation and angiogenesis assays.

**Microbiological Tests.** Five hundred milligram of the irradiated samples were aseptically transferred to a thioglycollate broth (Difco) and incubated at 30-32°C for 14 days. Turbidity in the medium indicates presence of microbial contaminants. The samples in the thioglycollate broth were plated on a Plate Count Agar (Difco) and incubated at 30-32°C for 2 days. The plate agar was also examined for colony growth. Determinations were done in duplicates for three sample lots.
**XTT-Based Cell Proliferation Assay.** T47D breast carcinoma cells (ATCC, USA) were maintained in RPMI 1640 medium with 10% heat-inactivated FCS, 1% penicillin-streptomycin and 1% fungizone (Gibco BRL). Cells were washed with PBS and dispersed in 0.05% trypsin. Cell suspensions were made with RPMI containing at 5% FCS (sub-optimal supplementation), 1% antibiotics and fungizone and the concentration was adjusted to 10,000 cells/ml final assay concentration. Cell cultures were plated into 96-well microtiter plates (50 ml/well) and were incubated with 50 ml of the amnion extracts at various concentrations (10, 20 and 30 mg/ml) in triplicates. After addition of the extracts, 100 ml of the XTT labeling cocktail (Boehringer Mannheim GMBH) was added. The optical densities were read at 450 nm after 17 hours. Cells without FCS and amnion supplementation served as negative controls while those with additional 5% supplementation served as positive control.

**Duck CAM Assay.** Fertilized 7-day old duck Anas luzonica (Fraser 1839) eggs were treated with 300 ml of amnion extracts through “windowing” and incubated for 3 days at 37°C in triplicates of two trials. The CAMs were dissected and photographed. Untreated eggs served as negative control while those injected with a prepared tumor extract served as positive control based on the premise that tumor extracts have angiogenic activities. Terminal blood vessels were examined under phase-contrast microscopy using LPO (100x).

**Results**

**Bioburden Status Before and After Irradiation**

The microbiological tests of irradiated amnions gave negative bioburden. It was interesting to note that the tests given before irradiation (after lyophilization) were often negative.

**In Vitro Cell Proliferation Assay**

When added to a confluent monolayer of T47D breast carcinoma cell line grown under a sup-optimal level of growth factor-supplementation with 5% FCS, water-soluble extracts of amnions significantly induced cell proliferation after assaying for 17 hours with XTT compared with cultures added with PBS alone (p<0.001). However, the cell proliferative capacity of human amnion did not significantly vary with radiation dose (Fig. 1).

**Induction of CAM Neovascularization**

Angiogenesis was induced upon addition of the human amnion extracts within 3 days. The results are summarized in Fig. 2 and individual representative photographs of the treatments are shown. It is observed that amnion induced-angiogenesis based

**Discussion**

During amniotic sac processing (after separation of chorion) special attention is paid to ensure that the epithelial side of amnion is placed directly on polyester net used as a support. After application on the wound, the epithelial side with the basement membrane is facing outwards; this will promote migration, attachment and spreading of the host cells encouraging epithelialization. The human amnion allografts are preserved by lyophilization or freeze-drying and subsequently radiation-sterilized with a dose of 25 kGy. Since the beginning of 1998 over 500 preserved RSHAM (with a total surface area over 50,000 cm²) have been prepared at PNRI and distributed to clinics and hospitals throughout Metro Manila (PNRI-DOST, 2001). While research on clinical application of RSHAM is being undertaken in various hospitals, we report our new findings on the basic science behind the wound healing activity of human amnions and how radiation processing of this placental material could possibly affect its biologic activity.

Cutaneous wound healing is a complex biological process leading to reestablishment of the epidermal barrier. In small wounds, the clinical significance of any differences in the rate of epithelization may be negligible, however, the role of timely wound closure is very critical in extensive wounds, such as burns (Scott-Connor et al., 1988), where the epithelization rates.
strongly correlate with morbidity and mortality. Motility and hyperproliferation of keratinocytes are among the major events during the re-epithelization and as was mentioned earlier, there are several peptide growth factors present in the amnion membrane that could initiate and sustain wound repair until the skin surface is fully epithelialized (Zeigler, Pierce & Herndon, 1997). The experiment on the XTT-based proliferation assay using T47D cancer cells demonstrates the general (cellular) mitogenic potential of amniotic membrane extracts. Lacking normal human fibroblasts and keratinocytes in our laboratory, we used instead T47 human breast carcinoma cells, a transformed epithelial line since the molecular basis of cell cycle is universal regardless of cell type, except that during carcinogenesis the checkpoints or “gatekeepers” are frequently deregulated (Peter, Bates & Parry, 1996). From our data, it can be deduced that the biologic activity of the amniotic mitogenic factors is unaffected even after radiation sterilization to 35 kGy. A possible explanation is the fact that there is a vast myriad of these growth factors with overlapping functions present in the placental material that any diminution in the cell proliferative activity accrued from radiation-induced molecular damages can be masked by redundancy and bioavailability of these mitogens.

Within a day, as the epithelial cells at the wound edge, e.g. in burn traumas, begin to proliferate, a basic synergism with induction of angiogenesis must be present to support the rapid transport of oxygen and nutrient to the nascent cells. Otherwise in physiologically normal conditions, angiogenesis virtually never occurs in adult tissues, except in the ovary, the endometrium and the placenta (Norrby, 1997). Angiogenesis is operative during embryonic development where the placenta is converted to a highly vascularized organ capable of mediating the implantation of the embryo and the efficient exchange of respiratory gases, nutrients and wastes between the maternal and the fetal systems (Ramsey, 1982) and by this virtue, it appears that amnions are a good source of angiogenic agents that can be applied for supportive care of wounds in dire need to be vascularized. The placenta secretes various hormones to induce placental vascularization, such as bFGF and angiopoietins (Reynolds & Redmer, 2001), and the recently cloned proliferin (PLF) and the proliferin-related protein (PRP) both belonging to the prolactin and growth hormone family, respectively (Jackon et al., 1994). We are not cognizant of previous studies that investigated individual radiosensitivities of known angiogenic agents, however based on our results of the standard CAM assay for angiogenesis, it is obvious that these amniotic angiogenic factors are more radiosensitive compared to those molecules for epithelial cell proliferation. It was proposed earlier that amniotic (epithelial) mitogenic factors are so numerous in the placental material that radiation-induced damage to cell proliferation activity is dampened; conversely, it can be inferred based on the target theory (Altman, Gerber & Okada, 1970) that the loss of angiogenic activity from amnions irradiated at 35 kGy may be due to fewer amounts and diversity of these placental angiogenic agents or those factors that can induce endothelial cell proliferation.

The choice of using 25 kGy as the generally accepted dose for sterilization of medical products is widely used to sterilize grafts (Philips, 1994). However the application of a 40% safety factor common for medical devices is considered excessive and would endow undesirable biochemical properties on the biomaterial (Tallentire, 1986; AAMI, 1991). While the Philippines utilize 25 kGy as standard dose, other countries, i.e.
Poland, uses 35 kGy (Tyszkiewicz et al., 1999) in their production of amnion allografts. This sterilization dose is estimated based on the probability of decimating a given population of contaminating microorganisms in order to achieve $10^{-6}$ sterility assurance level (SAL) since the graft is expected to come into contact with compromised tissues (IAEA, 1990). Based on results by the Polish Tissue Bank, amnions processed at a higher dose still maintain good take, adhere to wounds and persist even 3 weeks after grafting (Tyszkiewicz et al., 1999). While there is an advantage that the probability of microbial contamination is much less with the delivery of a higher radiation dose, our bioburden tests indicate that processed amnions prior to irradiation are already free from microbes. Other amnion tissue banks have also observed zero bioburden of freeze-dried amnions even prior to irradiation (Hai et al., 1992) and this may be attributed to good processing procedures and the presence of anti-microbial compounds in the amnion, such as allantoin (Hansbrough, 1992). From our experience, radiation processing at 35 kGy would already be an overkill and it is possible to lower the irradiation dose below 25 kGy to lessen the processing cost of the dressing since the starting material is already “sterile”. In addition, the reported adherence and “good take” of the amnion biologic dressings are a function of some structural molecules such dermal matrix proteins including collagen, tenascin, vitronectin and glycosaminoglycans as well as the lack of immunologic response (Hansbrough, 1987). This property was also investigated using molecular topographic examination and other physico-chemical analyses on irradiated membranes (Deocaris et al., 2004).

Finally, another issue that had recently become a concern is the transmission risks of HIV and Hepatitis B associated with human amnion tissue banking which thereby merits the use of higher radiation levels such as 35 kGy for graft sterilization. Additional precautions are currently being undertaken, i.e. pre-donor screening and washing of amnion with 0.05% sodium hypochlorite solution. These are integrated in the quality assurance system in tissue banks (Campbell et al., 1994), including that of PNRI to improve the safety features of RSHAM. In a tissue banking operation, the accepted levels of irradiation of RSHAM should consider all the given factors, namely, sterility requirements, viral transmission risks and more importantly, the radiosensitivity of amniotic wound healing properties as was addressed in this study.

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