

## Evaluation of the trophic effect of human placental polydeoxyribonucleotide on human knee skin fibroblasts in primary culture

O. Muratore<sup>a,\*</sup>, A. Pesce Schito<sup>a</sup>, G. Cattarini<sup>a</sup>, E. L. Tonoli<sup>a</sup>, S. Gianoglio<sup>a</sup>, S. Schiappacasse<sup>b</sup>, L. Felli<sup>b</sup>, F. Picchetta<sup>b</sup> and G. C. Schito<sup>a</sup>

<sup>a</sup>*Institute of Microbiology, University of Genoa, Largo Rosanna Benzi 10, I-16132 Genoa (Italy),  
Fax +39 10 50 4837*

<sup>b</sup>*Chair of Orthopaedics and Traumatology, University of Genoa, Largo Rosanna Benzi 10, I-16132 Genoa (Italy)  
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**Abstract.** A simple assay capable of evaluating the trophic effect of growth factors or active principles on human skin diploid fibroblasts in primary culture has been developed. The results indicate that at physiological concentrations (20–100 µg/ml) a human placental polydeoxyribonucleotide (PDRN) preparation enhances the growth of human skin diploid fibroblasts of the knee in primary culture. This effect is consistently reproducible in the case of patients over 60 years of age, and may explain previously reported data on the successful clinical applications of human placental preparations, suggesting a selective benefit of PDRN in wound healing when compared to other treatments.

**Key words.** Human placental polydeoxyribonucleotide (PDRN); primary tissue culture; human skin fibroblasts; wound healing.

Recent progress in the understanding of the biochemistry of human placenta [1–7] has induced several authors to reconsider the rationale; first put forward by Filatov in 1948, for the clinical application of placental extracts in different diseases, including gastric ulceration [8], uveitis [8] and gynaecological disorders [8]; semi-greasy placenta ointments had been successfully used in the treatment of acute first or second degree radiodermatitis [9]. In 1984, a clinical paper showed a selective benefit with respect to other treatments of a preparation obtained from human placentas, in wound healing [10], in aerated alopecia and even in psoriasis [10]. In 1990, Viganò and collaborators successfully utilized a human placental polydeoxyribonucleotide (PDRN) preparation in the cure of second degree burns [11]. Recently, several authors demonstrated that vasculopathic patients treated with a PDRN preparation displayed a significant increase in the speed of healing of the ulcerative lesions of the lower limbs [12].

Fibroblasts [13] play a central role in wound healing by producing and maintaining the connective tissue matrix [14–18] and eventually contracting the newly formed connective tissue to bring together the edges of the wound [16, 17]. The present work has defined a simple assay to evaluate the kinetics of growth of human skin diploid fibroblasts in primary culture. Employing this newly developed assay, PDRN has been shown to be capable of promoting cell doubling and spreading of human knee skin fibroblasts, particularly in older patients, thus providing a firmer rationale for its utilization in efforts to accelerate wound healing.

### Materials and methods

The cell cultures were maintained in a humidified incubator, at 37 °C with 5% CO<sub>2</sub> in air, in Minimum Essential Medium with Earle's salts (MEM, Gibco, Paisley, Scotland) supplemented with 15% fetal bovine serum (FBS), 1% non-essential amino acids (Flow, Irvine, Scotland), L-glutamine (292 mg/l) and gentamycin (160 mg/l).

A human placental polydeoxyribonucleotide (PDRN) preparation in aqueous solution and NaCl (PLACEN-TEX, Mastelli, Sanremo, Italy) was dissolved directly in complete growth medium at various concentrations. The PDRN constitutes more than 80% of the dry part of the purified drug. Moreover, since the human placenta secretes a variant of pituitary growth hormone (hGH-V) [19] which can promote the growth of dermal fibroblasts [20], it is important to show that the PDRN preparation does not contain biologically active proteins, but only free amino acids (<12%), residual very low molecular weight peptides, and glycosaminoglycans (<8%). In fact, this drug has been obtained by the manufacturer using an intensive denaturation procedure using proteolytic enzymes and other treatments. Moreover, a sterilization process (121 °C for 15 min) occurs as the final step of drug isolation.

According to previously published procedures, the optimal concentration of PDRN to be added to primary explant cultures was determined by analysing the effects of various concentrations of the PDRN (from 100 to 2,000 µg/ml) on the growth kinetics of cloned MRC-5 fibroblasts (fig. 1), examining the developing monolay-

\* Corresponding author.

ers and cell morphology daily for nine days under different magnifications (from  $10\times$  to  $400\times$ ) in triplicate control and treated cultures, using a light microscope for tissue cultures (Wilovert, Will Wetzlar GmbH). Briefly,  $15 \times 10^4$  cloned MRC-5 fibroblasts were seeded in 2 ml of complete growth medium in 35 mm Petri dishes. Special care was taken to ensure uniform distribution in all culture dishes at the beginning of each experiment. Twenty-four hours after plating, the cells were re-fed, and thereafter given fresh medium on the fourth and seventh day with complete growth medium and PDRN at various concentrations. Cell counts were made on cell suspensions with a Coulter counter and verified with a Bürker hemocytometer at the time of seeding (day 0) and daily for nine days, after trypsinization of treated and control triplicate cell cultures (fig. 1). The counts in triplicate dishes differed by less than 5% throughout the experiments.

Thirty-two skin explants were obtained from 16 male and 16 female patients, who ranged from 6 to 93 years of age and were undergoing surgical operations of the knee. All skin explants measured about  $2 \text{ cm}^2$  and were put in

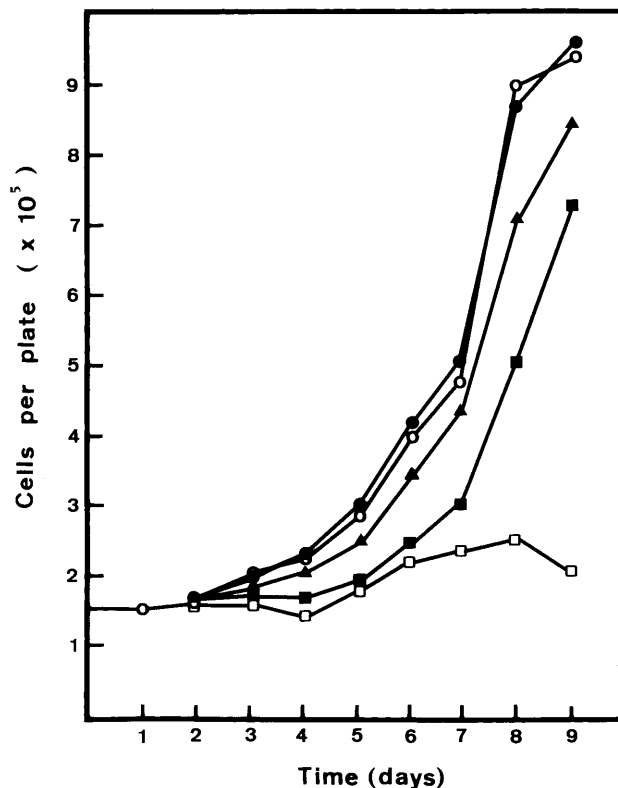


Figure 1. Effects of various concentrations of the PDRN (● 100, ▲ 500, ■ 1,000 and □ 2,000  $\mu\text{g/ml}$ ) on the growth kinetics of cloned MRC-5 fibroblasts. Twenty-four hours after plating, the cells were re-fed, and given a medium change on the fourth and seventh day with complete growth medium and PDRN at various concentrations. Treated and control (○) triplicate cell cultures were trypsinized and counted with a Coulter counter at the time of seeding (day 0) and daily for nine days. The counts in triplicate dishes differed by less than 5% throughout the experiments.

a sterile test tube placed on ice, containing 20 ml of complete growth medium, in an effort to remove surgical disinfectant. To obtain primary cell cultures, each skin explant was transferred in a sterile 35 mm Petri dish (Corning, N.Y., U.S.A.), chilled and containing 1 ml of complete growth medium to avoid cell dehydration. Then, each explant was finely chopped and the almost microscopic pieces of skin (=fragments) were transferred to a second sterile test tube placed on ice, by repeated washing and pipetting over the surface of the dish, using complete growth medium and a sterile glass pipette previously modified in such a way as to enlarge the entry diameter slightly.

In this way a suspension of the fragments was prepared and seeded in two equal parts (3 ml per well) onto the culture surface of two wells (base surface =  $3.8 \text{ cm}^2$  per well) of a twelve-well tissue culture plate (Costar, U.S.A.). The first well was utilized as control culture, the second (=test well) to evaluate the trophic effect of PDRN.

Before seeding, the suspension was homogenized using a vortex, to be sure that controls and test wells contained skin explants of equal size at the start of the experiment. This is very important, because the number of cells present in the wells correlates with the explant size at the start, having a major impact on outgrowth, proliferation, and spreading.

Finally, the twelve-well tissue culture plates were placed in a humidified incubator at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  in air to allow the sedimentation of the fragments. Two hours after seeding, growth medium was gently removed by suction and the fragments were re-fed with complete growth medium (2 ml per well) and PDRN in appropriate concentration and replaced to incubate.

Cell attachment to the plastic/liquid interface is essential to promote cell migration and the proliferation of fibroblasts [16, 17, 21]. The experiments carried out during the first part of the present work suggested the introduction of a sterile 'gauze disk', just larger than the base surface of the well and octagonally-shaped, to facilitate its placing within the well and on the pieces of each explant (fig. 2). In fact, the lipidic mass present in the skin adipocytes of some donors appeared so abundant (fig. 2) as to keep the fragments floating and prevent their attachment to the base surface of the wells.

Twenty-four hours after seeding, a careful microscopic inspection of control and treated cultures generally revealed the absence of free fibroblasts (fig. 2). The presence of single fibroblasts, mechanically released from the stroma of the fragments during the procedures of chopping, was a rare event. Moreover, a careful microscopic inspection was carried out daily throughout each experiment.

Usually, the onset of growth followed the migration of fusiform fibroblasts from the stroma of the fragments

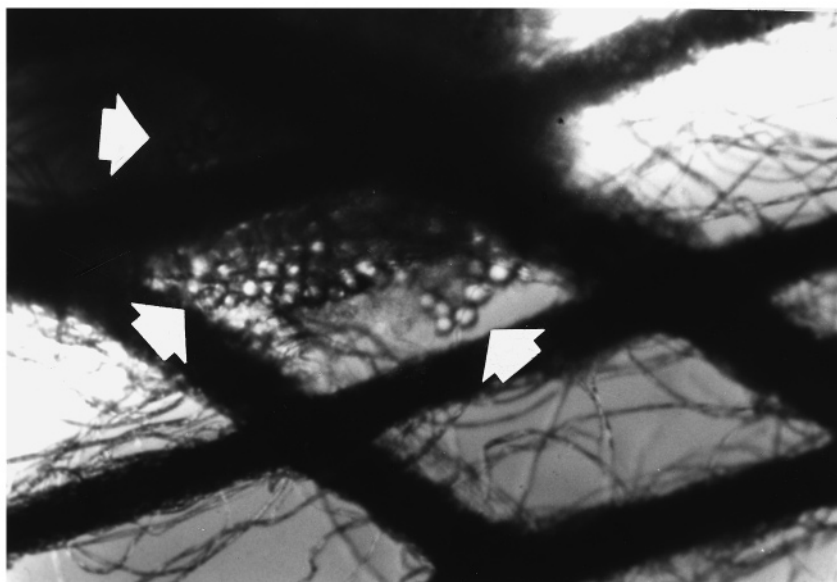


Figure 2. Piece of a skin explant (=fragment) of the knee entrapped by the grid of a sterile 'gauze disk' ( $\times 40$ ). The gauze disk, not much larger than the base surface of the well, was adjusted within the well and on the pieces of each explant. White arrows indicate a very abundant number of skin adipocytes. Without 'gauze disk', the lipidic mass of the adipocytes could prevent the attachment of the fragments to the base surface of the well.

and became manifest by the presence of cells at different stages of the mitotic cycle, i.e. single rounded cells and/or not yet separated developing daughter cells (eight-shaped) and/or well-separated rounded daughter cells. The cells at different stages of the mitotic cycle were always present together with a few fusiform fibroblasts and appeared rather darker than the latter.

## Results

The optimal concentration of PDRN to be added to primary explant cultures was determined on cloned MRC-5 fibroblasts. No cytotoxic effect was noted in the first three hours with all the dilutions used. After nine days of culture, concentrations of 500, 1,000 and 2,000  $\mu\text{g/ml}$  affected MRC-5 cell growth, inhibiting the formation of a confluent monolayer with a marked reduction (over 75%) of the viable cells in the case of 2,000  $\mu\text{g/ml}$  (fig. 1). In the presence of concentrations of 100–150  $\mu\text{g/ml}$  the monolayer morphology and confluency ( $9.5 \times 10^5$  cells per plate) appeared identical to those of the control cultures (fig. 1). For this reason, a concentration of 100  $\mu\text{g/ml}$  was finally added to primary explant cultures (17 cases). A concentration of 20  $\mu\text{g/ml}$  was subsequently used to evaluate the effect of lower concentrations of PDRN (15 cases).

Without 'gauze disk', when a primary culture of fibroblasts was started, the cell migration and proliferation stopped after a few doubling cycles (fig. 3) in 8 out of 8 wells in total (table 1). The utilization of a sterile 'gauze disk' on the pieces of the explants improved the percentage of spreading in control cultures (17 out of 22 cases

total = 77.3%) (table 2 and table 3), compared to the values observed in the control wells without this device (3 out of 10 cases total = 30%) (table 1). Furthermore, the 'gauze disk' significantly reduced the time necessary to obtain initiation of fibroblast growth in control cultures (average value = 9.8 days, compared to 20.7 days) (table 2 compared to table 1).

It is of interest to note that the fibroblast orientation is not determined by the 'gauze disk' stitch orientation (fig. 4). In fact, this observation may be utilized in further studies to go deep into the problem of the regulation of migration and orientation of human knee skin fibroblasts in primary culture.

The presence of PDRN facilitated the proliferation of fibroblast primary cultures, depending on its concentration. When 100  $\mu\text{g/ml}$  was used without 'gauze disk' in explants from two different donors, 66 and 87 years old respectively, the development of a primary culture was observed exclusively in the wells with PDRN (table 1). In the case of a 55 year old donor, the drug allowed fibroblastic proliferation to occur more quickly, i.e. 15 days earlier than in the control culture. With two other donors, the growth of fibroblast primary cultures started in the wells with PDRN at the same time of the controls, and in five cases growth of fibroblasts was not observed in any condition.

When a 100  $\mu\text{g/ml}$  dose was used with 'gauze disk', i.e. when an improved method was used, the PDRN allowed fibroblastic proliferation to occur more quickly, i.e. from 2 to 15 days earlier than in control cultures, in 5 out of 7 cases in total (table 2). Moreover, in one case the development of a primary culture was observed

Table 1. Effect of a 100 µg/ml dose of human placental polydeoxyribonucleotide (PDRN) on the patterns of growth of human skin fibroblasts of the knee in primary culture (without 'gauze disk').

Donor #	Growth detection		Days required for growth initiation		Donor age (years)
	PDRN concentration control	100 µg/ml	PDRN concentration control	100 µg/ml	
From 1 to 5	—	—	—	—	(from 13 to 55)
6	+	+	30	30	43
7	—	+	—	16	66
8	+	+	13	13	12
9	+	+	19	4	55
10	—	+	—	25	87

†The cell migration and proliferation stopped after a few doubling cycles in 8 out of 8 wells.

Table 2. Effect of a 100 µg/ml dose of human placental polydeoxyribonucleotide (PDRN) on the patterns of growth of human skin fibroblasts of the knee in primary culture (with 'gauze disk').

Donor #	Growth detection		Days required for growth initiation		Donor age (years)
	PDRN concentration control	100 µg/ml	PDRN concentration control	100 µg/ml	
11	+	+	12	9	29
12	—	—	—	—	16
13	+	+	6	4	45
14	+	+	8	4	22
15	+	+	18	3	93
16	—	+	—	4	74
17	+	+	5	2	34

Table 3. Effect of a 20 µg/ml dose of human placental polydeoxyribonucleotide (PDRN) on the patterns of growth of human skin fibroblasts of the knee, detected after forty days of primary culture (with 'gauze disk').

Donor #	Growth detection		Number of fibroblasts (in thousands)		Donor age (years)
	PDRN concentration control	20 µg/ml	PDRN concentration control	20 µg/ml	
From 18 to 20	—	—	0	0	from 24 to 70
From 21 to 25	+	+	350*	350*	from 6 to 38
From 26 to 28	+	+	200*	240*	from 16 to 62
From 29 to 31	+	+	250*	350*	from 48 to 81
32	+	+	300	390	57

\*Range of variability detected among different cultures of the same group = ± 5 (thousands).

Table 4. Summarizing data, correlated with the donor age, and obtained in the different experimental conditions, showing the influence of different doses of human placental polydeoxyribonucleotide (PDRN) on the patterns of growth of human skin fibroblasts of the knee in primary culture.

Range of donor age	Without 'gauze disk'		With 'gauze disk'	
	PDRN concentration 100 µg/ml		PDRN concentration 100 µg/ml	PDRN concentration 20 µg/ml
Over 60 years of age	+	+	+	+
From 20 to 60 years of age	i	i	+	+
Under 20 years of age	i	i	i	i

+ or i represent one case.

+ Benefit obtained with PDRN, and observed in respect of control cultures (whether as the development of a primary culture observed exclusively in the wells with PDRN, or as a proliferation quicker than the one observed in the control cultures, or as the number of fibroblasts exceeding by 20% to 40% the amount detected in controls after 40 days of culture).

i Identical situation observed in the wells with PDRN as the one observed in the control culture wells.

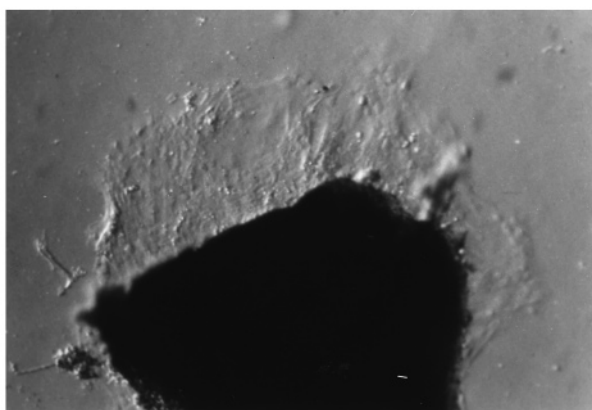


Figure 3. Development of a primary culture from a piece of skin explant of the knee (from a 66 year old donor) in the well with 100  $\mu\text{g}/\text{ml}$  of PDRN ( $\times 40$ ). Without 'gauze disk', the cell migration and proliferation stopped after a few doubling cycles.

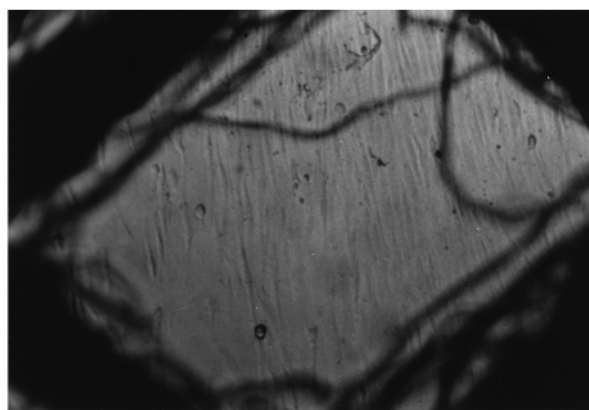


Figure 4. Example of intensive fibroblast migration and proliferation promoted by the 'gauze disk' in a control culture ( $\times 160$ ). Note that the fibroblast orientation is not determined by the 'gauze disk' stitch orientation.

exclusively in the well with PDRN, and only in one case growth of fibroblasts was not observed under any conditions (table 2).

When a 20  $\mu\text{g}/\text{ml}$  dose of PDRN was used with 'gauze disk', the trophic effect of the drug on the proliferation of fibroblasts was detectable only after 40 days of culture (table 3). After this period of incubation, treated and control cell cultures were trypsinized and cell suspensions were counted with a Coulter counter and a Bürker hemocytometer. In seven cases, the monolayers of the wells with PDRN showed a number of fibroblasts exceeding by 20% to 40% the amount detected in control cultures (table 3). In five cases, the number of fibroblasts in the wells with PDRN appeared identical to the one presented by control cultures, and in three cases growth was not observed under any conditions (table 3).

Above all, the data correlated with the donor age show that the benefit produced by PDRN on the growth of human skin fibroblasts of the knee in primary culture is very consistently reproducible (table 4) in donors over 60 years of age (8 out of 9 cases total). Moreover, the benefits appear reproducible in donors from 20 to 60 years of age (7 out of 13 cases total), particularly when the optimal concentration of PDRN (100  $\mu\text{g}/\text{ml}$ ) is used in combination with the most suitable methodology, i.e. in the experiments with 'gauze disk' (4 out of cases total). The benefit produced by PDRN appears to become inconsistent in the donors under 20 years of age (1 out of 10 cases total) (table 4).

## Discussion

The experiments carried out in the present work permitted the improvement of previously published procedures aimed at obtaining primary cultures of human diploid fibroblasts [21]. Moreover, a simple assay capable of evaluating the trophic effect of growth factors or active principles on human skin non-neoplastic fibro-

blasts in primary culture has been developed. A considerable amount of data have been published showing that normal human fibroblasts have a finite division potential when cultured in vitro [13, 21–29] and the growth of human diploid fibroblasts in primary cultures often requires the presence in the culture medium of promoters of neoplastic transformation [21, 30]. In many cases normal fibroblasts in primary culture are not able to reach confluency [21, 24] and the growth tends to stop after a few doubling cycles [13, 21, 24]. This behaviour has also been observed in our conditions (table 1 and fig. 3; traditional experiments without 'gauze disk'). The introduction of a sterile 'gauze disk' within the wells and on the fragments appeared to improve significantly the efficiency of the spreading of control primary cultures. This greater efficiency could be due to several different causes. First, the 'disk' makes easier the attachment of the fragments to the plastic/liquid interface, preventing their floating. Second, when a medium change is performed, the 'disk' breaks the turbulence produced, thus preventing the detachment of previously anchored fragments. Furthermore, the presence of the 'disk' promoted the migration of fibroblasts in the plane of the solid substrate (fig. 4), probably hindering the dispersion of auto-secreted growth factors [23, 24] and of extracellular matrix proteins [25–27]. For these reasons, the utilization of a 'gauze disk' seems very important in order to obtain a correct evaluation of the results when an exogenous growth factor or active principle, like PDRN, is being tested. In fact, the 'disk' ensures uniform distribution in the attachment of the fragments to the base surface of all culture dishes (control wells and test wells) at the beginning of each experiment and when a medium change is performed. Consequently, this uniform distribution of the fragments ensures uniform diffusion of auto-secreted growth factors, thus giving a more realistic impact on cell proliferation and spreading [21].

The results of the present study show that at physiological concentrations (20–100 µg/ml), PDRN enhances the growth of human skin diploid fibroblasts of the knee in primary culture, particularly in donors over 60 years of age (table 4). This effect may explain previously reported data on the successful clinical applications of human placental preparations, suggesting a selective benefit of PDRN in wound healing when compared to other treatments [10].

However, the trophic effect detected on human skin fibroblasts of the knee appeared to be significant but not intense (fig. 3, table 1 and table 3). Moreover, when a 20 µg/ml dose of PDRN was used, this effect was easily detectable only after a long term treatment (table 3). All these findings, and the significant percentage of test wells without growth of fibroblasts (9 cases out of 32 total), suggest that the effect occurs through physiological mechanisms not comparable to those induced by promoters of neoplastic transformation [21, 30]. This is an important feature that supports the clinical usage of PDRN.

The trophic effect expressed by PDRN may be attributed to the increased concentration of free nucleotides and nucleosides, as a consequence of the depolymerization of its molecule, produced by cellular nucleases [31]. This is in accord with previously reported data indicating that extracellular purine nucleosides and nucleotides are intracellular signals and potent mitogens for several types of cell, such as fibroblasts [32], endothelial cells [33] and neuroglia [34]. Several authors have demonstrated that extracellular adenosine and its nucleotides cause *synergistic* enhancement of DNA synthesis [35] in fibroblasts, when combined with polypeptide growth factors contained in serum and derived from platelets that are active in wound healing [21, 35].

Nevertheless, additional mechanisms to explain the trophic effect cannot be excluded. In fact, studies on the control of cellular proliferation *in vitro* have shown the importance of extracellular matrix components like the glycosaminoglycans [36], which are present in the PDRN preparation. Moreover, it is of interest to note that several authors demonstrated that deoxyribonucleosides added to cell cultures which had been exposed to UVC radiation [37] and solar radiation [38] were able to reverse the processes leading to chromosomal breakage. Finally, a recent paper shows that 'highly polymerized' mammalian DNA can be more effective than free nucleosides in increasing the survival frequency of lethally irradiated rodents [39]. Other authors found a DNA-binding protein on leukocytes and hypothesized that the receptor is present on every cell in the body except red blood cells [40]. The receptor may function primarily as a *salvage pathway*, suggesting that a range of nucleotide sizes binds the receptor: from naked DNA as small as 15 bp to the nucleosome frag-

ments that characterize apoptosis and signals that lead to cell survival and growth [40, 41]. Once bound, endocytosis of the receptor-ligand complex results in degradation of nucleotide and protein components [40]. These authors have traced some of this DNA to nucleotide pools in the cell nucleus and hypothesized that this is another labour-saving device that the body has evolved as a way of coping with immune stress [40]. Moreover, they suggested that some proportion of any antisense drug must pass through this pathway [40]. The trophic effect observed with PDRN on human skin fibroblasts in primary culture could also be due to this salvage pathway. The salvage pathway, i.e. a labour-saving device, could be utilized particularly in the case of senescent cells like the fibroblasts of old patients. Thus, the influence of the donor age on explant growth probably depends on the amplification of the trophic effect observed with PDRN on control cultures of not very viable and not proliferating fibroblasts.

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