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Ultraweak photon emission in assessing bone growth factor efficiency using fibroblastic differentiation

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Dedicated to the memory of Alexander Gurwitsch on the occasion of his 125th birthday.

Abstract

Photons participate in many atomic and molecular interactions and changes. Recent biophysical research has shown the induction of ultraweak photons in biological tissue. It is now established that plants, animal and human cells emit a very weak radiation which can be readily detected with an appropriate photomultiplier system. Although the emission is extremely low in mammalian cells, it can be efficiently induced by ultraviolet light. In our studies, we used the differentiation system of human skin fibroblasts from a patient with Xeroderma Pigmentosum of complementation group A in order to test the growth stimulation efficiency of various bone growth factors at concentrations as low as 5 ng/ml of cell culture medium. In additional experiments, the cells were irradiated with a moderate fluence of ultraviolet A. The different batches of growth factors showed various proliferation of skin fibroblasts in culture which could be correlated with the ultraweak photon emission. The growth factors reduced the acceleration of the fibroblast differentiation induced by mitomycin C by a factor of 10–30%. In view that fibroblasts play an essential role in skin aging and wound healing, the fibroblast differentiation system is a very useful tool in order to elucidate the efficacy of growth factors.

Keywords: Ultraweak; Photon; Fibroblast; Skin; Human; Ultraviolet; Growth factor

1. Introduction

At the beginning of this century, Alexander Gurwitsch, born 125 year ago in Russia, suggested that ultraweak photons transmit information in living systems [1], as summarized recently [2]. Although the results of Gurwitsch were refuted by Hollander and Klaus [3] and interest in this subject declined in the following decades, the presence of biological radiation was re-examined with the development of photomultiplier tubes in the mid-1950s by Facchini and co-workers [4]. In the 1960s, most of the work on ultraweak photon emission was performed by Russian scientists [5–7], while in Western countries several pioneers, Quickenden in Australia [8], Popp in Germany [9] and Inaba in Japan [10], independently developed methods for ultraweak photon measurements in a variety of different cells by the use of an extremely low noise, highly sensitive photon counting system which allows maximal exploitation of the potential capabilities of a photomultiplier tube. In the meantime it is commonly agreed that plant, animal and human cells emit ultraweak photons often called biophotons [11–17]. From these and additional investigations different origins for this very weak radiation have been proposed.

Most investigators think that very weak radiation results from radical reactions which can be produced by biological events such as lipid peroxidation. In studies of microsomal lipid peroxidation [18,19], it has been shown that the amount of malonaldehyde production and the intensity of emitted light are related to each other. Based on these studies, Inaba and co-workers proposed [20] that oxygen dependent light emission in rat liver nuclei was caused most likely by lipid peroxidation in the nuclear membrane. As discussed in detail by Cadenas and Sies [21], free radical decomposition of lipid hydroperoxides leads to the

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formation of excited chemiluminescent species by the self-reaction of secondary peroxy radicals formed from lipids, producing either singlet molecular oxygen or excited carbonyl groups.

However, there also exists a very interesting model, published in 1983 by Nagl and Popp [22], suggesting that there is a negative feedback-loop in living cells which couples together states of a coherent ultra-weak photon or biophoton field and the conformational state of the cellular DNA. The authors assume photon transfer or non-radiation chemical pumping from the cytoplasmic metabolism which results in changes of the DNA conformation via exciplex/excimer formation. Their hypothesis is based on experimental data reviewed by Birks [23] who also suggested these excimers as precursors of the pyrimidine photodimers, which play a key role in the radiation damage of DNA and have been most recently proposed as biomolecular signals in communication [24]. It was hypothesized that there exists an effective intracellular mechanism of photon absorption in normal human cells [25]. After UV-light irradiation of cells, pyrimidines absorb the energy in order to form pyrimidine dimers that could be responsible for influencing metabolic and cellular events, as was recently shown for photodimer induced melanogenesis [26].

Tilbury discussed in the multi-author review of van Wijk [27] that ultraweak photon emission has been detected in both the visible and ultraviolet region. Radiation in the visible region appears to be due to excited carbonyl groups and/or excited singlet oxygen dimers arising from lipid peroxidation, which in turn are associated with an increase in various reactive oxygen species such as the superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen. There is also substantial evidence for DNA playing a key role in these emissions as discussed above [9,28,29]. This macromolecule may especially be involved in the emission of ultraweak photons by the cell in the UV-region of the spectrum [27].

Recently, experiments with cultured human cells were reported [25] in which normal and DNA excision repair deficient Xeorderma pigmentosum of complemation group A (XPA) cells were UV-irradiated in Dulbecco's modified Eagle's medium (DMEM) and Earle's balanced salt solution (EBSS) and assessed for ultraweak photon emission. There was some evidence of induced photon emission from normal cells in Earle's balanced salt solution, but clear evidence of a fluence-dependent emission in XPA cells in medium and in EBSS were seen. Overall, it was clear that an important difference between normal and XPA cells was present and it is possible that XPA cells are less able to absorb ultraweak photons in contrast to normal cells.

Since Hayflick's pioneering work in the early 1960s, human diploid fibroblasts have become a widely accepted in vitro model system. Recently, Bayreuther and co-workers extended this experimental approach showing that fibroblasts in culture resemble, in their design, the hemopoietic stem-cell differentiation system [30]. They reported morphological and biochemical evidence for the fibroblast stem-cell differentiation system in vitro. They showed that normal human skin fibroblasts in culture spontaneously differentiate along the cell lineage of mitotic fibroblasts (MF) MFI–MFII–MFIII and post-mitotic fibroblasts (PMF) PMF IV–PMFV–PMVI–PMFVII. Additionally, they developed methods to shorten the transition period and to increase the frequency of distinct post-mitotic cell types using physical or chemical agents, such as mytomycin C, 5-fluorouracil, and 5-bromo-2-deoxyuridine, that have been reported to induce differentiation in a variety of cell systems, as we have recently summarized [31–36]. Mitomycin C is an effective chemotherapeutic agent for several cancers in man; this effect is probably related to the interaction with DNA leading to DNA–DNA crosslinks and DNA–protein crosslinks. We have previously demonstrated that the mitomycin C-treatment of three different normal human fibroblast strains (CRL 1221, GM 38, and GM 1717), frequently used in mutation, transformation, and aging research, induces characteristic morphological changes in the fibroblasts and brings about specific shifts in the [^{35}S]-methionine polypeptide pattern of total cellular proteins [31]. These results support the notion that mitomycin C accelerates the differentiation pathway from mitotic to postmitotic fibroblasts. Using this system, we were also able to demonstrate that no significant difference exists in the rate and the extent of the excision-repair response to thymine-containing pyrimidine dimers following UV-irradiation shortly after mitomycin C treatment of distinct strains of human skin fibroblasts and in the mitomycin C-induced PMF stage of these cells [31]. In addition, aphidicolin inhibits excision repair of UV-induced pyrimidine photodimers in low serum cultures of mitotic and mitomycin C-induced postmitotic fibroblasts of human skin [32]. In view of the essential role fibroblasts play in skin aging and wound healing, our results imply that the fibroblast differentiation system is a very useful tool in order to elucidate the efficiency of growth factors.

2. Material and methods

2.1. Growth factor samples

Three different lots of bovine bone growth factors (BP2, BP3, and BP5) in cryotubes (lyophilised; 1 mg/tube) were provided by Jeff Moehlenbruck (Sulzer Biologics, Austin, TX, USA). In the following experiments, factors BP2, BP3, and BP5 were tested at a concentration of 5 ng/ml. The mitotic cells were treated for 1 week, while the postmitotic cells were incubated with these growth factors over 2 weeks. The growth factors were also added during treatment with mitomycin C to put the cells into a post-mitotic status.

2.2. Cell culturing

Skin fibroblasts (GM05509A) derived from a 17-year-old female patient affected with Xeroderma pigmentosum of complementation group A (XPA) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ, USA). XPA cells derived from a 10-year-old female (CRL 1223) were purchased from the American Type culture collection (Rockeville, USA). The cells were cultured in tissue culture plastic flasks (surface=75 cm^2, Gibco Basel, Switzerland) in 15 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco, Basel Switzerland) supplemented with 10% fetal calf serum and 100 units ml^{-1} penicillin–streptomycin as previously described [37]. XPA cells were plated 1:3 and passages 15–17 were used. Postmitotic fibroblasts were prepared as previously described [33]. For the determination of ultraweak photon emission in mitotic fibroblasts, 3×10^5 cells were seeded into 10-cm diameter tissue culture dishes and grown for 1 week until confluency with and without growth factors at a density of 5 ng/ml. UVA-irradiated fibroblasts and control cells were frozen gently in liquid nitrogen in DMEM from which phenol red had been omitted. For the determination of ultraweak photons in postmitotic fibroblasts, 1.5×10^5 cells were seeded into 10-cm diameter tissue culture dishes. After 48 h the medium was removed and a new complete medium containing 2×10^{-7} M mitomycin C with and without growth factors was added for 24 h. The medium was then removed and new complete DMEM with and without growth factors was added for 4 days. The cells were additionally treated with 2×10^{-7} mitomycin C, essentially as described elsewhere [37]. The medium was removed and new complete DMEM with and without growth factors was added for 7 days until they have been in a postmitotic stage. Control cells which had been immediately treated with mitomycin C were also used. The cells were counted in a haemocytometer from Neubauer (Flow Laboratories, Baar, Switzerland) and cells were counted in triplicates ($\pm$10%). Cells were frozen in liquid nitrogen using the cryopreservation apparatus from Biotech Research Laboratories (Rockeville, MD, USA). Controlled gradual temperature reduction during the cryopreservation was critical for the maintenance of cell life and viability. This apparatus preserves cells by cooling at the rate of 1°C per min when placed in a −70°C freezer. After 5 h or overnight, the frozen samples were transferred to the liquid nitrogen transport storage system. Since frozen cell samples using this procedure survive also without DMSO, as checked by tryptan blue coloration, no DMSO was added.

2.3. Irradiation facilities for UVA radiation exposure

Before irradiation, the cells were rinsed twice with 3 ml of phosphate-buffered saline (PBS), covered with 4 ml of PBS and were irradiated as monolayers in tissue culture dishes at room temperature. For UVA radiation a UVASUN 3000 lamp (Mutzhas, Munich, Germany) was used at a dose rate of 300–600 Wm^{-2} at a distance of 60–90 cm. Irradiation periods were on average 10–45 min maximum. The spectral output of the lamp was analyzed with a calibrated Optronic model 742 spectroradiometer (Optronics Laboratories, Orlando, FL, USA). Before and after each experiment radiation fluences were monitored by an International Light Radiometer (IL 1700, calibrated against the spectroradiometer). The emission spectrum was between 340 and 450 nm with a maximum at 380$\pm$ 30 nm and the cells were then frozen in liquid nitrogen as described above until measurements on ultraweak photon emission were carried out.

2.4. Ultraweak photon determinations

For ultraweak photon emission measurements, different fibroblasts samples were transported in liquid nitrogen. Some cells were transported fresh in T-75 flasks and used for ultraweak photon emission. The cells were thawed and were diluted in a volume of 10 ml of Dulbecco's modified Eagle's medium, from which phenol red was omitted, and were transferred to a quartz sample glass (inside dimensions=2.2×2.2×3.8 cm^3; wall thickness=0.15 cm). Detection and registration of spontaneous and light-induced emitted photons was accomplished at 25°C, as described before [25]. The test samples were kept in a dark chamber in front of a single photon counting device equipped with an EMI 9558 QA photomultiplier tube (diameter of the cathode=48 mm; temperature=−25°C). This high-sensitivity photon counting device is described in detail by Popp and co-workers [9,38] and measures photon intensities as low as 10^{-17} W in the range of 220–850 nm. Signal amplification is normally 10^6–10^7 and the dark count ranges between 10 and 15 counts per second (cps). Maximum efficiency of the S20-cathode is 20–30% within the range of 200–350 nm (maximum is at 250 nm), decaying almost linearly down to 0% at a wavelength of 870 nm and mean quantum efficiency in the entire spectral range is about 10%. The integral intensity values within each interval of 40 ms was stored and processed by an interfaced computer. For light induced emission experiments, irradiation of the test sample was performed perpendicular to the detecting direction with focused white light from a 75-W Xenon lamp. Each measuring cycle was started by irradiating the sample for 5 s. The measurements for the monochromatic light induction was performed by changing the spectrum of the inducing light through a monochromomator (PTI, Hamburg, Germany) from 300 to 450 nm (25 nm interval). The irradiation intensity of the monochromator reaching the sample in the range of 350–450 nm was linearly increased from 0.1 to 0.6 Wm^{-2} as measured by a radiometer.

Table 1
Percentage of cell counts in immediately trypsinized monolayer fibroblasts with and without treatment with growth factors[a]

Differentiation stage of cells	Control	BP 2	BP 3	BP 5
Mitotic fibroblasts (%)	100	100	140	120
MMC-treated mitotic fibroblasts (%)	100	100	160	120
Postmitotic fibroblasts (%)	100	133	133	155

[a] A total of 100% correspondend to 3.0×10^6 mitotic fibroblasts (controls and miotomycin C-treated) and 0.9×10^5 postmitotic fibroblasts. Growth factor efficiency=BP3>BP5>BP2.

3. Results and discussion

3.1. Bone factor induced proliferation in fibroblastic differentiation

The proliferation of fibroblasts was determined by counting cultured cells following a 1–2-week treatment with 5 ng/ml DMEM of each lot of bone growth factor. The percentage of the increase of cell proliferation was between 0 and 60% as shown in Table 1.

3.2. Ultraweak photons in mitotic and postmitotic human skin fibroblasts from patients with Xeroderma pigmentosum (XPA)

We performed ultraweak photon experiments in the fibroblast differentiation system with human skin fibroblast from Xeroderma pigmentosum patients (GM 05509A; CRL1223). Bayreuther and co-workers [30–36] showed biochemical and morphological evidence for the fibroblast differentiation system in vitro. They showed that normal human skin fibroblasts in culture spontaneously differentiate along the cell lineage of mitotic (MF) and postmitotic fibroblasts (PMF). Additionally, they developed methods to shorten the transition period and to increase the frequency of distinct postmitotic cell types using physical agents such as ultraviolet light (UV) and mitomycin C (MMC). To differentiate the human skin fibroblasts we have used mitomycin C. Mitotic skin fibroblasts are small in size in contrast to postmitotic cells, which are enlarged by a factor of up to ten [33]. As shown in Fig. 1 there is no discernible difference between untreated mitotic fibroblasts, miotomycin C treated cells, and MMC-induced postmitotic cultures 1 week following MMC-treatment in age-matched XPA cells (GM05509 A; CRL 1223; 4×10^7/cells).

We previously found that postmitotic fibroblasts are significantly reduced in UV-induced ornithine-decarboxylase responses [33]. In skin fibroblasts from a patient with Xeroderma pigmentosum (XPA) we detected a slight increase in the rate of thymine dimer induction formed after UV-irradiation of the MMC-induced postmitotic stage 3 weeks after MMC-treatment [31]. For ultraweak photon emission, our experiments with cultured human cells in which normal and XPA cells were UV-irradiated showed an important difference between normal and XP cells for the absorption of photons [25]. Since the white light induction for ultraweak photon emission in the differentiation system with XPA cells was very similiar in both cell

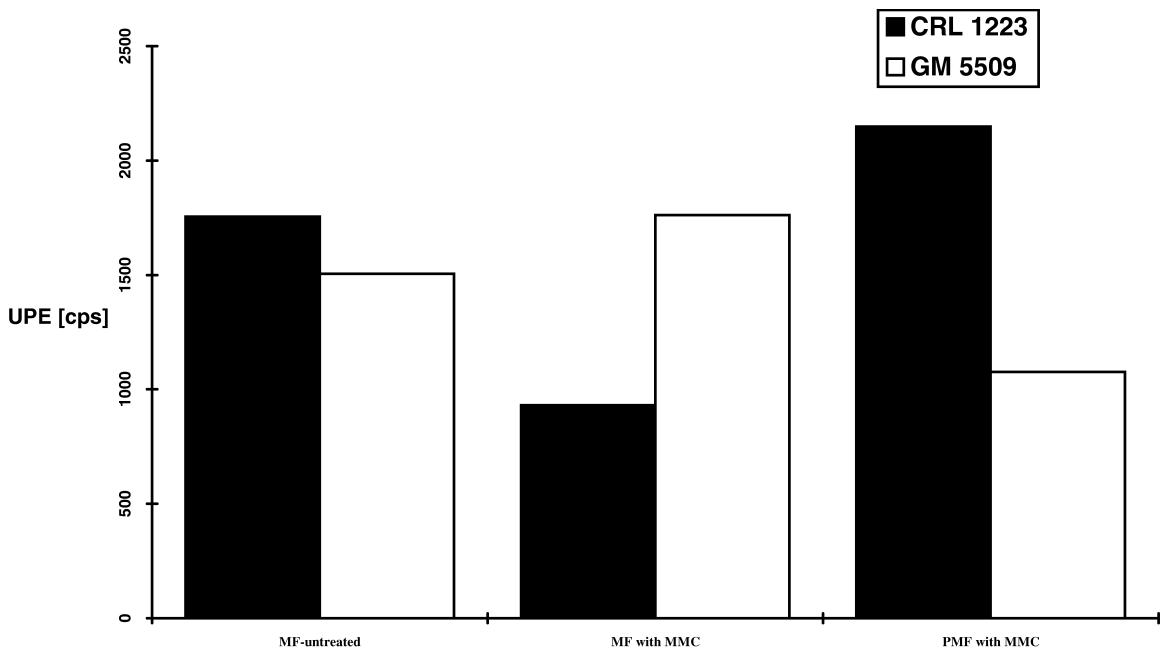

Fig. 1. Total counts of photoinduced ultraweak photon emission registered in the first second after illumination with white light for 30 s of XPA human skin fibroblasts. Background values obtained with medium alone are subtracted. Average of two independent experiments in triplicate. Standard deviation is ±10%.

lines (see Fig. 1), we decided to test the growth factors with the still commercially available skin Xeroderma Pigmentosum fibroblasts originating from the Human Genetic Mutant Cell Respository (XPA; GM05509A).

In previous experiments with normal cells, we found that white light-induced photon emission dynamics are identical after successive irradiations of approximately 1-min intervals, and even several cycles of illumination and measurement do not quantitatively change the emission intensity for several hours [28]. The light-induced emission curves are hyperbolic as we reported most recently with mouse melanoma cells [29]. These results confirm several previous investigations in plant and mammalian cells [11,15,16]. As interpreted by Li and Popp [38,39], the hyperbolic decay kinetics found in living systems after pre-illumination with white light may indicate coherence of ultraweak photons due to collective excitation of nucleic acids within the DNA of the investigated fibroblasts.

In the first experiments with human XPA skin fibroblasts it was found that measurable amounts of ultraweak photons can be achieved with cells densities as low as $2–3\times10^4$ cells/ml (data not shown). At these cell densities, control values after irradiation with a white light source for induction of ultraweak photons were determined in mitotic and postmitotic fibroblasts from fresh cells and compared to those of frozen cells. The results are shown in Table 2.

Although there was a small increase in the induced photon emission from fresh cells, most probably due to the stress condition of the transport, it can be concluded that the frozen cells showed the same type of emission. Similiar results were obtained for monochromatic induction of the fibroblasts in the range of 300–450 nm as shown for MMC-mitotic cells in Fig. 2.

In a previous report [25], we found a slight increase in the dark count rates in postmitotic fibroblasts in XPA cells in contrast to normal fibroblasts. Therefore, the dark count rate at a cell density of 3.6×10^4 cells/ml in untreated and growth factor treated postmitotic fibroblasts was determined (data not shown) and this increase of the dark count rate in postmitotic cells was confirmed. From these dark count rates a decrease percentage of the accelaration of MMC-induced differentiation could be measured and it can be concluded that the growth factors reduce the accelaration of differentiation in post-mitotic cells induced by MMC by a factor of 10–30% (data not shown).

As shown in Fig. 2 the most significant increase in ultraweak photon emission after monochromatic irradiation of fibroblasts between 300 and 450 nm was found at 350 nm. Therefore, the induced photon emssion after 350-nm light induction at a cell density of 3.6×10^4 cells/ml in untreated and growth factor treated MMC-mitotic fibroblasts was determined. In addition, the cells were treated with 400 kJ/m^2 UVA as described elsewhere [37]. For mitotic cells, the results are shown in Table 3 showing an improvement of photon absorption ability in XPA-cells for the growth factors in the efficiency range of BP5>BP3>BP2. For MMC-treated mitotic fibroblasts similiar results have been obtained as shown in Table 4 as well as for MMC induced-postmitotic fibroblasts (data not shown).

We have previously found that after white light induction there is a small decrease from mitotic to postmitotic fibroblasts from XPA-cells, while there is in MMC-treated normal fibroblasts a significant increase [28]. Additionally, we detected that normal cells absorb UV-photons more efficiently than XPA-cells [25]. As shown in Table 4, growth factor treatment indeed increased the monochromatic induction in these cells similar to normal cells. Most interestingly, UVA irradiation in mitotic and postmitotic cells significantly reduced the monochromatic induction of ultraweak photons. It can be assumed, therefore, that the decrease in mitotic untreated and MMC treated cells, which have been exposed to growth factors, is due to the absorption of photons, emphasizing a return to normal basal status.

To finally summarise, our results indicate that the tested growth factor solutions have an effect on growth and ultraweak photon emission in the differentiation model of human skin fibroblasts and provides a new and powerful non-invasive tool for the development of skin science. Its high sensitivity can be applied in all fields of skin research, in investigating skin abnormalities and in testing the effect and efficacy of regenerative, anti-aging, and UV-light protective agents at very low concentrations.

4. Abbreviations

DMEM	Dulbecco's modified Eagle's medium
EBSS	Earle's balanced salt solution
MF	Mitotic human skin fibroblasts
MMC	Mitomycin C
PMF	Postmitotic human skin fibroblasts
UVA	UV-light from 320 to 400 nm

Table 2
White light induced ultraweak photon emission (counts per 40 ms) in immediately trypsinized monolayer fibroblasts (fresh) compared to fibroblasts transported in liquid nitrogen (frozen)[a]

Differentiation stage of cells	Induced emission (fresh)	Induced emission (frozen)
Mitotic fibroblasts	72	54.2±5.4 (N=6)
MMC-treated mitotic fibroblasts	74	40.0±5.0 (N=6)
Postmitotic fibroblasts	54	33.8± 3.4 (N=6)

[a] 10 ml of DMEM.

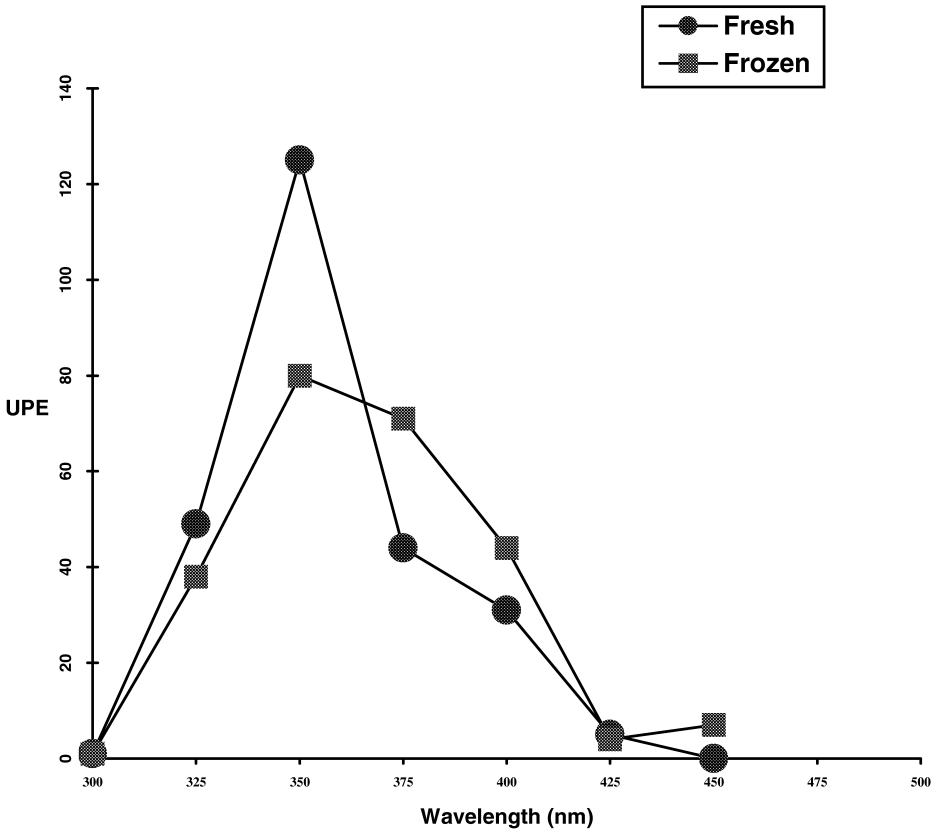

Fig. 2. Monochromatic irradiation (300–450 nm) induced ultraweak photon emission (UPE; counts per 10 ms) in immediately trypsinized monolayer fibroblasts (fresh) compared to fibroblasts transported in liquid nitrogen (frozen). Average of triplicate determinations are given. Standard deviation is less than 10%.

Table 3
350 nm monochromatic irradiation induced ultraweak photon emission (%) in mitotic fibroblasts with and without treatment with growth factors in controls and UVA-treated cells (400 kJ)[a]

Differentiation stage of cells	Control	BP 2	BP 3	BP 5
Mitotic fibroblasts				
Control	100	50	80	185
UVA-treated	100	47	0	10

[a] Averge of triplicate determinations are given. Standard deviation is less than 10%. Growth factor efficiency=BP5>BP3>BP2.

Table 4
350 nm monochromatic irradiation induced ultraweak photon emission (%) in MMC-treated mitotic fibroblasts with and without treatment with growth factors in controls and UVA-treated cells (400 kJ)[a]

Differentiation stage of cells	Control	BP 2	BP 3	BP 5
MMC-treated mitotic fibroblasts				
Control	100	161	320	106
UVA-treated	100	0	0	33

[a] Average of triplicate determinations are given. Standard deviation is less than 10%. Growth factor efficiency=BP3>BP2=BP5; All very efficient.

UPE Ultraweak photon emission
XPA Xeroderma pigmentosum skin fibroblasts of complementation group A

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