# BIOLOGICAL RESPONSES OF NIH 3T3 TO 193 NM EXCIMER LASER IRRADIATION. COMPARISON WITH 254 NM IRRADIATION.

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## 1. INTRODUCTION

During conventional UV radiations, activation of genes is tightly linked to the presence of DNA damages. At 254 nm, the major cellular chromophore is the nuclear DNA, with cyclobutane pyrimidine dimers being the major photoproduct (1,2). At 193 nm, DNA is more strongly absorbing than at 254 nm. However, the quantum yields of the photoproducts induced at these two wavelengths are different (3) and damage to cellular DNA in the form of pyrimidine dimers or single-strand breaks was reported to be only marginal or undetected (4,5). Peptide bonds, many amino acid side chains, insaturated lipids, esters, and other cellular molecules absorb at 193 nm (6,7). Consequently, the most of the energy is thought to be absorbed by proteins, leaving the DNA shielded. About 60 % of the radiation can apparently be blocked by 1 µm of cytoplasm (8). However, exposures of cultured human fibroblasts to subablative doses of 193 nm laser radiation resulted in changes of genes expression such as collagenase, metallothionein and *c-fos* (9). The mechanisms by which the 193 nm radiation affects gene expression are not known. The site of primary interaction of the radiation could be different from the site of the genetic response. Thus, the signal transfer could pass through the cytoplasm via the nucleus. One hypothesis is that cytokines may regulate the transduction pathway event. By example, the TNF-alpha which is induced by UV-radiations (10). More, it can activate transcription factors such as AP-1 or *c-fos* and stimulate the growth of normal fibroblasts (11). Moreover, TNF-alpha plays a major role in the inflammatory processes by enhancing the remodeling of extracellular matrix (12,13) in which mainly matrix metalloproteinases and collagenase participate. Besides, matrix metalloproteinases are responsive to cytokines and particularly the 92 kDa gelatinase (gelatinase B or metalloproteinase 9 or MMP 9) is induced and regulated by TNF-alpha (14).
To understand the cellular response to high energy laser radiation, we investigated cell proliferation and cell activation. The results showed a decrease of the cell proliferation and an increase of the cell activation. These variations are more marked after the irradiation at 193 nm. In addition, we have quantified the variations of TNF-alpha and gelatinase B in supernatants of fibroblasts irradiated with 193 nm laser radiation or 254 nm conventional UV radiation. The results showed that both wavelengths caused increased rate of TNF-alpha and of the gelatinase B (expressed under two forms, latent and active) but the laser was significatively more efficient.

## 2. MATERIAL AND METHODS

### 2.1. *Cell cultures*

The mouse fibroblasts NIH 3T3 cells obtained from American Type Culture Collection were grown in RPMI 1640 medium containing 10% of decomplemented fetal calf serum (FCS - Boehringer Mannheim), 20 mM L-glutamine and 100 µg of antibiotic mixture (penicillin, streptomycin, neomycin - Gibco-BRL) at +37°C in 5% $CO_2$. Two days before irradiation, $1.5 10^6$ cells were plated into 8.5 cm diameter plastic tissue culture dishes (Falcon). Prior to irradiation, the medium was removed and the cells rinsed with phosphate-buffered saline (PBS-Boehringer Mannheim). Irradiations as well as sham exposures of controls (zero dose) were performed in the absence of any solution, with plates held either vertically in the case of laser irradiations or horizontally in conventionnal UV irradiations. After irradiation, cells were plated for 8 hours into 96-well plates at $10^5$ cells /well, with 200 µl medium per well.

### 2.2. Irradiations

Laser irradiations were performed with a SEL 520 SOPRA excimer laser at 193 nm (ArF), with nominal pulse duration of 13 ns. Laser beam was defocussed to illuminate an 8.5 cm diameter cell culture plate (56.7 $cm^2$) held vertically. The energy per pulse, measured with a joulemeter Molectron JD2000 detector, was 57 mJ/pulse, corresponding to a peak irradiance of $7.7 10^8$ $W/m^2$. Cells were exposed to fluences of 10, 50 and 120 $J/m^2$ by varying the number of pulses delivered with 1 Hz repetition rate. Irradiations at 254 nm was performed with a Bio-Sun system (Vilbert-Lourmat) where UV energy, delivered by 3 UV-VL20 lamps with a constant irradiance of 4 $W/m^2$, is controlled by microprocessor.

### 2.3. *Cell proliferation*

Cell proliferation was assessed by (methyl-$^3$H) thymidine incorporation.

### 2.4 *Cell activation*

A tetrazolium colorimetric assay, using the dye MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide], measures the reducing ability of mitochondrial enzymes.

### 2.5 *Quantification of total proteins*

Total proteins was quantified according to the sensitive method described by Bradford (15).

2.6 *Quantification of TNF biological activity in culture medium*
TNF-alpha secretion was measured by a biological activity test using actinomycin-D-treated L-929 cells as described by Fish and Gifford(16).
2.7. *Gelatin zymography*
Gelatinolytic activity was performed in cell supernatants as described by Heussen and Dowdle (17).
2.8. *Statistical analysis*
It was performed according to Student'st-test for unpaired data (StatView II software, Abacus concept Inc., Meylan, France). $p < 0,05$ was considered significant.

3. RESULTS
3.1. *Number of cells*
The number of cells was measured immediately and 8 hours after the irradiation. At 193 nm, the number of cells decreased sharply in a dose-dependent manner. The most pronounced decrease was observed 8 hours after the exposure. At 254 nm, a similar dose-dependent decrease was measured 8 hours after irradiation. A high mortality of cell (36%) was detected at 120 J/m$^2$ at 193 nm just after the irradiation, a smaller one was seen (15%) 8 hours later at the same dose. In opposite, at 254 nm no mortality was observed at any dose just after the irradiation and 8 hours post-irradiation, the cell mortality detected was at 8%.
3.2. *Cell proliferation and activation*
The proliferation of NIH 3T3 cells is markedly reduced, reaching about -75% at 120 J/m$^2$ after each kind of irradiation. In return, both wavelengths induced a dose-dependent cell activation in irradiated cells but the augmentation was about 2.4-fold greater after 193 nm laser exposure than after 254 nm irradiation at 120 J/m$^2$.
3.3. *Quantity of total proteins*
The wavelength of 193 nm induced a dose-dependent increase of the amount of total proteins in irradiated cells. Even though at 254 nm the increase was not significant between 50 and 120 J/m$^2$. The increase was 1.6 times greater after 193 nm laser irradiation than after 254 nm at 120 J/m$^2$. Irradiations induced cycloheximide-dependent increase amount of protein.
3.4. *TNF-alpha and Gelatinase B activity* (Figure 1)
A large dose-dependent increase was observed after 193 nm irradiation. In contrast after 254 nm irradiation, no increase was detectable except for the fluence of 120 J/m$^2$ but the TNF activity was 2 times lower. Both irradiations at 193 and 254 nm cause a significant and dose-dependent increase of both latent and active forms of excreted gelatinase B. The proportion of the two forms was identical but the augmentation was about 2.1-fold greater after 193 nm laser exposure than after 254 nm irradiation at 120 J/m$^2$.

4. DISCUSSION
To our experimental conditions, an important cell mortality is induced only by 193 nm laser radiation. This suggests that radiation damage induced by laser 193 nm exposure differs from that of 254 nm radiation. At 193 nm, the maximal contribution of radiation injury to cell death was observed immediately after irradiation. Cells either are too injuried and died within minutes, or are not damaged enough and survive after repair. At 254 nm, a wavelentgh which is known to produce DNA damages, no immediate cell death was observed and mortality was still low 8 hours later. The nuclear damages occuring at 254 nm irradiation do not induce an immediate cell death. Then, the cell mortality observed within minutes after irradiation may likely originate from an alteration of the cell membrane. The high absorption by cytoplasm and membrane proteins may account for the damages induced by 193 nm photons and the high cell mortality observed immediately after laser exposure. The decrease of the cell proliferation is important after 193 nm radiation. Cell activation which was really important after 193 nm irradiation, as shown by the cycloheximide dependent increment of total proteins. This suggests that cells have tried to repair their damages. This effect on cell activation is different from the one published by Kochevar and al, (18) who found a dose-dependent inhibition of the MTT reduction. Cell activation is increased and could be due to the increment of TNF-α which is involved in gene expression regulation and proteins synthesis. Indeed TNF-α ellicit a rapid induction of *fos* mRNA (19) and, it is well known that activation of *fos* is part of a typical response of mammalian cells to UV-C light (20). Our results confirm this *fos* induction which occur in fibroblasts following irradiation at 193 nm. The level of TNF-α could be considered as a parameter permiting to evaluate the damaging effect of 193 nm photons on the plasma membrane because it may be related to the change of arachidonic acid metabolism occurring in the altered plasma membrane (21,22). Kochevar and al, (18) have demonstrated an increase in cell membrane permability associated with a release of arachidonic acid within 15 minutes after exposure to 193 nm radiation whereas exposure to 254 nm radiation did not cause this release. They suggest that plasma membrane damage induced by 193 nm radiation was obvious from the release of arachidonic acid. This release of arachidonic acid observed by Kochevar and al, and the secretion of TNF-α in cell supernatants measured in our experiments seem to occur at approximately the same fluences (23) shown that arachidonic acid which is also released in response to TNF, induced *c-fos* mRNA. Furthermore Gelatinase B, involved in the degradation process of basement membrane, is more expressed after laser exposure, supposed to be changing the cell adhesion process. The results indicated that the 88 kDa active form which is involved in the TNF-α activation was specifically increased after 193 nm laser irradiation. However, the quantification of TNF-α biological activity in cell supernatant could be originated from the membrane compartment release from

damaged cells by 193 nm photons. The Gelatinase B increase could be also induced by extra cellular TNF-α but cell activation observed with the lowest fluence suggest that the origin of the quantified TNF-α did not only issue from membrane compartiment of dead cells. Moreover, the 88 kDa active form of Gelatinase B contribute to increase the quantity of TNF-α produced in surviving cells.
Thus, the results suggest that the plasma membrane is the site of the primary damage in cells exposed to 193 nm laser radiation. The mechanism of interaction between 193 nm photons and the plasma membrane is unknown but the primary effects of the 193 nm radiation are a cell activation, a more important expression of Gelatinase B and of TNF-α. This latter cytokine could be the signal of induction from the plasma membrane to the nuclear transcription factors of genes whose expression was specifically changed by 193 nm laser radiation.

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5. REFERENCES

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Figure 1 : Gelatinase B activities and TNF-alpha biological activity in cell supernatants, 8 hours after excimer laser irradiation at 193 nm (a) or UV conventional irradiation at 254 nm (b).
(*** p < 0,0007 ; ** p < 0,006 ; * p < 0,04)