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Novel approaches towards a detailed control of the mechanism and efficiency of photosensitized processes in vivo

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Abstract

The photoexcitation of an endogenous or exogenous biological chromophore can result in the re-emission of light as fluorescence or phosphorescence; this may be used for photodiagnostic applications or in the conversion of at least part of the absorbed radiation into thermal or chemical energy. The last two processes generate photothermal and photochemical sensitization respectively leading to the irreversible damage of cells and tissues; hence they can be used for phototherapeutic purposes. The efficacy and scope of photodiagnostic and photother-apeutic techniques are improving as we increase our knowledge about the influence of biological microenvironments on the mechanisms of photoprocesses and as new approaches are developed for controlling the biodistribution of photosensitizing agents in vivo and the depth/diffusion of incident light into tissues. In particular, even the localized and sublethal damage of cells can often be modulated to generate intracellular signalling in order to either induce a programmed cell death (apoptosis) or an acceleration of cell metabolism (photostimulation).

Keywords: Control; Efficiency; Mechanism; Photosensitized processes

1. Introduction

The control of photoprocesses occurring in in vivo systems presents a challenging problem. In the first place, the multiplicity of endogenous constituents of cells and tissues which can absorb selected wavelengths in the UV and visible spectral range (see Table 1 for a list of the main chromophores which are present in mammalian tissues) makes it difficult to achieve a high spatial confinement of the photodamage [1]. Moreover, the photosensitivity of a biological system can be enhanced by the presence of exogenous compounds (''photosensitizers''), which are introduced into the organism through the diet, via interaction with the environment or in order to induce a predetermined alteration of the system [2]. The situation is further complicated by the typically compartmentalized structure of most biological systems. As exemplified in Table 2, the nature of the microenvironment can markedly affect both the physicochemical properties of the electronically excited states of a molecule (e.g. by modulating its aggregation state) and the efficiency of a photoreaction, such as the photo-oxidation of tryptophan, an amino acid residue which often plays a critical role in the biological activity of proteins [3,4]. Finally, we must not forget the presence of several biological, chemical or physical amplification factors, which may propagate the photoeffects to sites which are at a significant distance from the site at which light is initially absorbed [5].

In spite of these difficulties at both the theoretical and experimental levels, the field of photobiology is rapidly evolving towards an advanced control of the selectivity and efficiency of in vivo photoprocesses owing to the significant developments which have recently been made in the elucidation of the optical properties of mammalian tissues [6], as well as in laser technology, fast spectroscopic techniques in the time-resolved and time-gated domains [7] and the predetermination of the biodistribution and subcellular partitioning of externally added photosensitizing agents [8]. On this basis, the utilization of photobiological approaches for diagnostic and therapeutic purposes is undergoing a steady expansion, thereby broadening the scope of photomedicine.

2. General principles

As shown in Fig. 1, when a molecule undergoes electronic excitation, several photophysical steps follow, which can eventually result in three processes: (1) the re-emission of light, namely fluorescence or phosphorescence, which is generally important for diagnostic purposes; (2) the conversion

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Table 1
Main endogenous chromophores in biological systems

Biomolecule	Chromophore	Absorption range (nm)	Comments
UV light absorbing			
DNA, RNA	Purine and pyrimidine bases	220–310	n,π* and π,π* transitions peaking at 260–270 nm
Proteins	Indole (tryptophan)	250–310	The overall absorption is dominated by tryptophan (largest ε)
	Phenol (tyrosine)	240–290	
	Benzene (phenylalanine)	240–270	
Urocanic acid	Imidazole (deaminated histidine)	220–280	Mainly acts as a photoprotective agent
NADH	Nicotinamide	320–350	Protonation–deprotonation processes with NAD are possible
Visible light absorbing			
Haemoproteins	Tetrapyrrolic macrocycle of porphyrin	400 (Soret band) plus smaller intensity Q bands between 480 and 650	The position/intensity of bands is dependent on the presence of a metal ion and position of peripheral substituents
Chlorophylls	Partially reduced tetrapyrrolic macrocycle	400, 500–600, 660	Differ from porphyrins for the high intensity red band
Bilirubin	Linear tetrapyrrole	420–500	Peaks at 460 nm
Flavins	Isoalloxazine ring	400–480	Highly photolabile through formation of radical species
Carotenes	Polyene	400–520	Largely acts as a photoprotective agent
Collagen, elastin	Glyco amino acids	380–450	Broad, largely featureless spectrum
Melanin	DOPA-derived polymer	200–1100	Non-structured spectrum with absorbance steadily decreasing from short to longer wavelengths

of at least a fraction of the absorbed light energy into heat (non-radiative decay), which may lead to a substantial increase in the temperature of the microenvironment of the chromophore; (3) the promotion of a photochemical reaction due to the higher reactivity of the photoexcited molecule. Events (2) and (3) are expected to cause permanent damage of the system, which can be suitably manipulated in order to achieve a therapeutic effect.

In several cases, photochemistry becomes effective only when molecular oxygen is present. Such photoprocesses are defined as "photodynamic" [9] and can occur via two competitive pathways (Fig. 2): (1) energy transfer from the initially excited chromophore (endogenous or exogenous) to a suitable acceptor, most frequently oxygen, which is then promoted to a very reactive and cytotoxic species, called singlet oxygen; (2) electron or hydrogen transfer between the photoexcited chromophore and a nearby substrate, with the generation of radical intermediates, which are efficiently scavenged by oxygen leading to peroxy derivatives. Several UV- and visible-absorbing photosensitizers have been proposed as phototherapeutic agents, of which psoralens are widely used for the treatment of some skin diseases (e.g. psoriasis and vitiligo) in association with UVA irradiation [10]. However, most frequently, photosensitizers exhibiting absorption bands in the red spectral region (600–900 nm) are selected for phototherapeutic applications owing to the

Table 2
Effect of microenvironment on photophysical and photochemical processes

Fluorescence emission of haematoporphyrin (Hp)		
System	Fluorescence lifetime (ns)	Monomerization (%)
2 μM Hp, buffer pH 7.4	15.4, 3.9	93.5
75 μM Hp, buffer pH 7.4	14.9, 3.8	78.5
2 μM Hp, aqueous SDS	16.8	100.0
75 μM Hp, aqueous SDS	16.8	100.0
100 μM Hp, 10% methanol	15.7, 4.2	83.3
100 μM Hp, 100% methanol	11.9	100.0
Rate constant for reaction of $^1O_2$ with L-tryptophan		
System		Rate constant (10^7 M^{-1} s^{-1})
D_2O, pH 7.4		7.2
10% D_2O, 90% methanol		1.3
10% D_2O, 90% formamide		8.7
Albumin, buffer pH 7.4		1.8

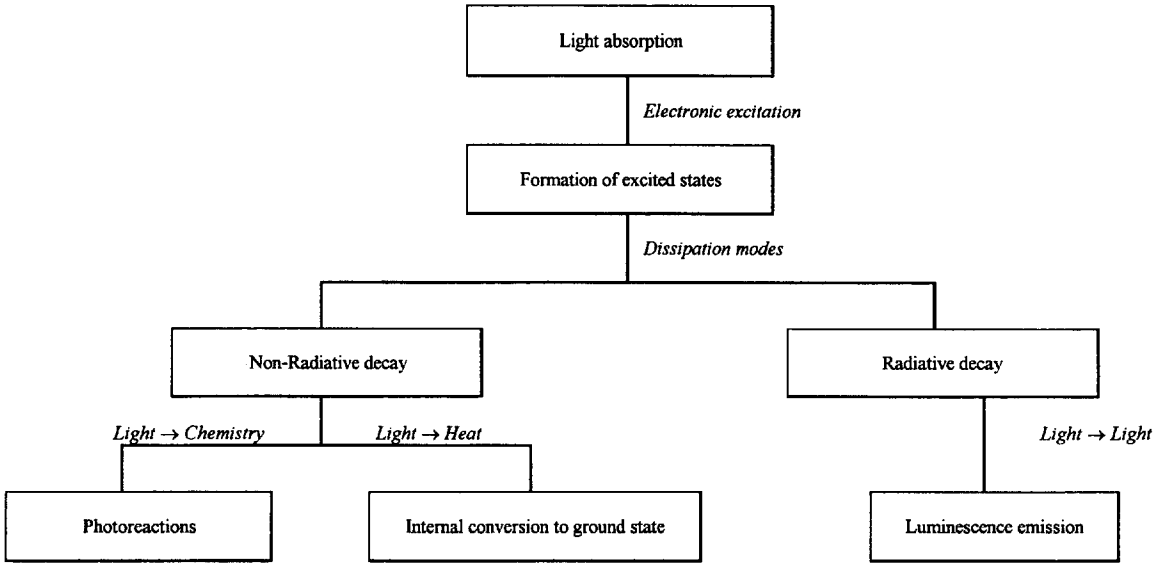

Fig. 1. Photophysical and photochemical processes subsequent to electronic excitation of a molecule.

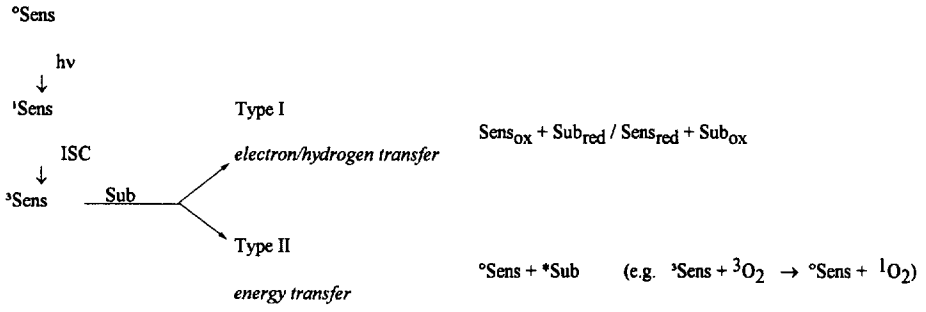

Fig. 2. Schematic representation of type I and type II photosensitization mechanisms.

Table 3
Second generation tumour photosensitizers involved in phase II/III clinical trials

Photosensitizer	Formulation	Absorption maximum (nm)
Benzoporphyrin derivative	Liposome	680
Sn(IV)-etiopurpurin	Liposome	670
Zn(II)-phthalocyanine	Liposome	673
N-Aspartyl-chlorin e_6	Aqueous solution	674
m-(Tetrahydroxyphenyl)-chlorin	PEG/ethanol/water	650

PEG, polyethylene glycol.

greater penetration power of these light wavelengths into mammalian tissues and the lack of competing absorption by endogenous tissue constituents, which minimizes the risk of widespread photosensitivity [11].

In general, both direct and sensitized photoreactions originate from the long-lived lowest triplet state (Fig. 2). Therefore optimal photodiagnostic agents are characterized by a high quantum yield of radiative decay from the first excited singlet state with a concomitant inefficient intersystem crossing to or very fast decay from the triplet state. Biological systems include several classes of endogenous fluorophores emitting in the UV (e.g. proteins, nucleic acids) and visible (e.g. flavins, chlorins or porphyrins) ranges; once again, fluorescent compounds which can target specific sites in cells or tissues can be artificially introduced into an organism [12]. Selected examples of the three above-mentioned photodiagnostic and phototherapeutic applications are given below.

3. Photodynamic therapy

Photodynamic therapy (PDT) is based on the property of a photosensitizing dye to be accumulated in larger amounts and/or to be retained for longer periods of time by tumour tissues compared with peritumoral tissues [13]. The most frequently used photosensitizer for the PDT of tumours is a chemically prepared derivative of haematoporphyrin, which is known under the commercial name of Photofrin II [14];

Table 4
Correlation between LDL transport of tumour photosensitizers (Sens) and selectivity of tumour targeting

Photosensitizer	LDL bound (%)	Sens ratio tumour/ peritumoral tissue
GePc-OAc	24.8	2.61
GePc-Chol	27.2	5.68
GePc-Et	29.5	7.37
ZnPc-Chol	33.0	8.11
GePc-Hex	35.7	8.48
GePc-Dec	46.9	11.81

LDL, low density lipoproteins; ZnPc, Zn(II)-phthalocyanine; GePc, Ge(IV)-phthalocyanine; axial ligands: Chol, bis-(diphenylcholesteryloxy-siloxy); OAc, bis-(dimethyl-butanoylacetate-siloxy); Et, bis-(triethyl-siloxy); Hex, bis-(tri-*n*-hexylsiloxy); Dec, bis-(tri-*n*-decylsiloxy).

however, a few second generation photosensitizers, which are structurally related to porphyrins, but show a markedly enhanced absorbance in the red, are now in phase II/III clinical trials (Table 3) [14]. Thus the illumination of a neoplastic lesion with light specifically absorbed by the photosensitizer at an appropriate post-injection time interval causes efficient tumour necrosis. This phototherapeutic modality has been applied to a variety of solid tumours [15], although it appears to be particularly useful for the treatment of inoperable malignancies as well as of infiltrating tumours. Of course, any further development of the technique requires an adequate control of the selectivity of tumour targeting by the photosensitizer; this goal can often be pursued by associating the photosensitizing agent with a suitable delivery system [14], including monoclonal antibodies (MABs) directed against antigens which are present at the surface of malignant cells or serum low density lipoproteins (LDLs) which are known to exhibit a preferential interaction with rapidly proliferating cells via receptor-mediated endocytosis.

For the MAB–sensitizer complexes, very high degrees of specificity have been obtained with regard to the labelling of malignant cells in vitro [16]; however, when applied to experimental tumours in animals, the method is limited by the low number of photosensitizer molecules which can be delivered within the neoplastic lesion without impairing the specific recognition of the MABs by the antigens typical of the tumour. Moreover, the overall efficacy of the photoprocess is reduced by the fact that the photosensitizer is external to the tumour cell. As a consequence, this approach is particularly promising for the early diagnosis of neoplastic lesions, even of small size, owing to the high sensitivity of presently available spectroscopic assays aimed at detecting the fluorescence emission from the tumour-loading dye [17].

Some of these drawbacks can be circumvented by the use of LDLs as endogenous carriers [18]. LDLs incorporate hydrophobic or amphiphilic photosensitizers into their lipid moiety; up to 150–200 porphyrin molecules per LDL molecule are released inside tumour cells in a cyclic process, undergo partitioning in subcellular membranes and once photoexcited induce a highly preferential necrosis of malignant cells. Several porphyrins and their analogues have been shown to become selectively associated with serum lipoproteins after systemic injection via lipid-type vehicles (liposomes, oil emulsions, inclusion complexes) and the selectivity index (ratio of photosensitizer concentration in the tumour to the peritumoral tissues) can often be correlated with the percentage of photosensitizer which is bound to LDLs (see Table 4); in particular, in the case of haematoporphyrin, the pre-incorporation into LDLs markedly increases the efficiency of tumour targeting compared with the same dye administered in physiological solution.

Other photosensitizer delivery systems have been proposed [19] with an aim to enhance the specificity of tumour loading, including the epidermal growth factor, polylysine or polyethylene glycol and nanoparticles. The potential of these approaches still needs to be assessed since few experimental results have been reported so far.

4. Photothermal sensitization

Of the endogenous chromophores, haemoglobin, melanin and water have been used as photothermal sensitizers due to their ability to undergo very efficient non-radiative decay from their electronically excited states to the ground state [20]. As can be seen from Table 5, the absorption of IR light by water is mainly used for the removal of large tumour masses by surgical lasers. On the other hand, haemoglobin and melanin are most usefully excited by laser sources operated in the pulsed regime; if the pulse frequency is shorter than the thermal relaxation time of the target, the photogenerated heating of the biological system cannot appreciably diffuse to the surroundings and a high spatial confinement of the photodamage can be achieved [21].

Table 5
Examples of photothermal sensitization

Light-absorbing chromophore	Irradiation wavelength (nm)	Irradiation regime	Observed biological effects
Oxyhaemoglobin	577	0.3 μs	Specific destruction of blood vessels
Melanin	600	8.5–40 ns pulses	Selective injury of melanosomes
Water	1064	30 ns pulses	Vaporization of tumour mass
Malachite green	620	10 ns pulses	Inactivation of dye-bound enzyme

The thermal relaxation time depends on a variety of factors, especially in the case of complex biological structures; however, to a first approximation, this parameter can be assumed to be proportional to r^2, where r is the area of the irradiated target. On this basis, portwine stains, which are characterized by abnormally dilated blood vessels (diameter larger than 100 μm), can be specifically removed by irradiation with pulsed 513 nm light from an argon laser or 580 nm light from a flash-lamp-pumped dye laser (corresponding to one absorption band of haemoglobin), since normal capillaries have a diameter of 10–50 μm with a thermal relaxation time in the 0.1 μs range.

The photoexcitation of melanin has been used for the selective destruction of melanosomes in pigmented melanoma [22]. Since melanin pigments display a continuous absorption throughout the UV and visible spectral interval, irradiation can be performed at various wavelengths, thereby achieving different penetration depths into the illuminated tissue. It must be emphasized that photothermal processes are by no means restricted to endogenous tissue constituents, since several dyes are known [20] to be non-fluorescent and to be devoid of any appreciable photodynamic activity, and hence to possess the basic photophysical properties for inducing photothermal sensitization. Such dyes include cyanine, triphenylmethane and azo derivatives, as well as porphyrins, chlorins and phthalocyanines coordinated with paramagnetic metal ions, which drastically shorten the lifetime of the triplet state. It has been shown [23] that pulsed laser irradiation of malachite green, a triphenylmethane dye, complexed with different enzymes, causes a loss of enzymatic activity as a consequence of thermally induced denaturation of the protein three-dimensional structure.

Although photothermal sensitization has been investigated to a limited extent so far, this technique appears to possess great scope and potential since it shows unique features which could integrate the existing phototherapeutic modalities in several cases and take full advantage of recent developments in the field of optoelectronic techniques. Such features include:

the lack of dependence of at least the initial photoprocesses on the presence of oxygen, which could be useful for the treatment of poorly vascularized tissues and/or necrotic tissue areas;

the possibility to promote photoexcitation in the far-red/near-IR region, where the penetration of light into both lightly and heavily pigmented tissues is maximal, and several low-cost and simple diode laser sources are now commercially available;

the absence of persisting general skin photosensitivity, which represents a major side effect of photodynamic therapy, since sunlight-photoactivated porphyrinoids are often retained for several weeks in cutaneous areas;

the modulation of the photodamaged targets and nature of the photoinduced modification of the system through a suitable selection of irradiation parameters and photogenerated temperature increase.

5. Photosignalling

A novel approach to photobiological selectivity is attracting increasing attention on the basis of a more detailed definition of the mechanisms by which photodynamic sensitizers (either endogenous or exogenous) generate cell inactivation. When sublethal cell damage occurs, as it is often induced by low concentrations of cell-bound photosensitizers and/or low light dosage (e.g. a few J cm^{-2} at a fluence rate of 1–20 mW cm^{-2}), a cell response to the photosensitized stress is often elicited; such a response involves the activation of one or more signalling events leading to either propagation of the initially confined damage to distal sites (which may be critical for cell functioning and survival) or stimulation of cell metabolism with repair of the photodamage and accelerated cell growth. The sequence of steps responsible for photosignalling [24] has not been elucidated completely; however, signal transduction is certainly the result of the integration of a variety of pathways (Fig. 3): transcriptional activation, release of mediators such as cytokines and histamine and second messengers such as members of the family of *ras* proteins, biosynthesis of stress proteins and gene expression have been identified [25]. In particular, PDT has been shown [26] to cause tumour necrosis by both random cell death, i.e. as a consequence of the irreversible modification of multiple targets which are essential for cell metabolism, and apoptosis, namely programmed cell death where initial damage of membrane components, such as steroids or unsaturated lipids, represents the signalling process which eventually leads to rapid cell inactivation through DNA fragmentation. On the other hand, singlet oxygen- or superoxide anion-mediated oxidative damage of a limited number of endocellular sites and changes in the redox properties of specific components of the mitochondrial respiratory chain have been proposed [27] to be responsible for the repeatedly observed, albeit poorly understood, process of low intensity red-light-induced stimulation of cell proliferation.

These findings open up new prospects in the field of phototherapy. Thus apoptosis could become an important tool for enhancing the efficacy of photodynamic treatment, especially at the level of deep-sited tissue layers at which the intensity of the incident light is strongly reduced, as well as for cells which are relatively distant from blood capillaries and hence accumulate smaller concentrations of systemically injected photosensitizing agents. At the same time, photobiostimulation is being used as an anti-inflammatory and analgesic treatment with reportedly favourable results [28].

6. Conclusions

Traditionally, photomedicine has been focused on the treatment of cancer and dermatological diseases. It is now obvious that several new fields of application are becoming accessible. Photomedical methodologies have the intrinsic advantage of dual selectivity, since the photodamaging proc-

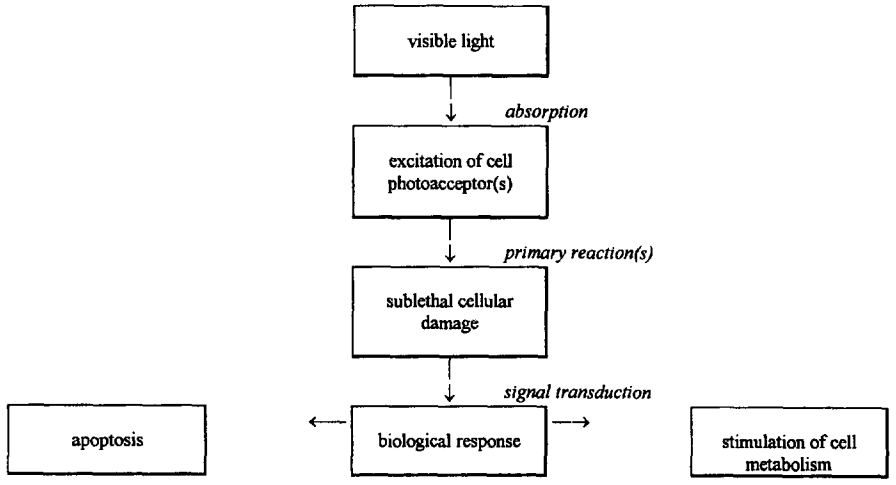

Fig. 3. Schematic representation of photosignal transduction leading to either cell damage (apoptosis) or cell stimulation (activation of selected metabolic pathways).

ess is the result of the combined effect of two factors (the light wavelength and the photoactive chromophore), each of which has no cytotoxic activity. Thus, even if an externally introduced photosensitizer is retained in a variety of tissues, no adverse undesired effect is induced in healthy areas provided that the incident light beam is centered on the diseased tissue and the photosensitizer is cleared from the organism in a reasonably short period of time. Furthermore, the extent of the photodamaged area can be modulated by the choice of irradiation wavelength, so that tissue layers located beyond the penetration depth of the selected photons are spared from damage even when potentially photoreactive species are present therein.

For several applications, broad-band emitting lamps are adequate; interference filters can be used if narrower spectral ranges are required. However, much of the recent progress in the field of photomedicine has been promoted by the availability of tunable and diode laser sources, which are significantly cheaper than many of the originally used medical or surgical lasers. While the coherence of light is generally unnecessary for in vivo applications, the monochromaticity and the possibility of efficient coupling of the light beam with optical fibres are unique and important features of lasers.

Finally, as the parameters optimizing the efficacy of the various phototherapeutic approaches are defined in detail, a synergistic interaction between the single modalities can be envisaged. As mentioned above, the outcome of PDT treatment for tumours can be potentiated by a simultaneous induction of random necrosis and photosignalled apoptosis of neoplastic cells. Moreover, a combination of photodynamic and photothermal sensitization has recently been reported [29] to enhance remarkably the extent of necrotic areas in pigmented melanoma.

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