

Clinical biomarkers and imaging for radiotherapy-induced cell death

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Abstract

Introduction Radiotherapy, like most anticancer treatments, achieves its therapeutic effect by inducing different types of cell death in tumors.

Cell death markers and imaging modalities To evaluate treatment efficacy a variety of routine anatomical imaging modalities is used. However, changes in tumor physiology, metabolism and proliferation often precede these volumetric changes. Therefore, reliable biomarkers and imaging modalities that could assess treatment response more rapidly or even predict tumor responsiveness to treatment in an early phase would be very useful to identify responders and/or avoid ineffective, toxic therapies. A better understanding of cell death mechanisms following irradiation is essential for the development of such tools.

Cell death and available assays In this review the most prominent types of radiation-induced cell death are discussed. In addition, the currently available assays to detect apoptosis, necrosis, mitotic catastrophe, autophagy and senescence *in vitro* and, if applicable, *in vivo*, are presented.

Keywords Cell death · Apoptosis · Radiotherapy · Annexin V · Imaging · Biomarker

1 Introduction

1.1 Cell death

Cell death is an essential biological process for eliminating abundant and unwanted cells during embryonic development, growth, differentiation and maintenance of tissue homeostasis. Deregulation of cell death pathways is associated with various diseases. Failure to eliminate cells that have been exposed to mutagenic agents may contribute to the development of cancer and resistance to anticancer therapy. In fact, the capacity to evade (apoptotic) cell death has been defined as one of the hallmarks of cancer [1].

It has become clear in recent years that cell death comprises a large number of different types of cellular demise (Table 1). According to a recent classification at least eight different types of cell death can be defined [2]. These can be divided into apoptotic and non-apoptotic. The major types are discussed in this review.

1.1.1 Apoptotic cell death

Apoptosis is defined by morphological criteria and by the requirement for active participation of the dying cell (Table 1). It can be induced by a great variety of stimuli, including growth factor deprivation, cell detachment (also known as *anoikis*), cytostatic drugs and ionizing radiation. Such stimuli may use unique “private induction pathways” to engage the apoptotic machinery. However, the molecular events in the execution phase of apoptosis are shared and impinge on the caspase family of proteases. Caspases are synthesized as pro-enzymes and activated by proteolysis. The private induction pathway activates the inducer caspases; these in turn cleave and activate the effector caspases, whose proteolytic action on specific cellular

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Table 1 Morphological and biochemical characteristics of the major types of cell death and their detection/imaging methods

Type of cell death	Morphological changes			Biochemical features	Detection/imaging methods	
	Nucleus	Cell membrane	Cytoplasm		<i>In vitro/ex vivo</i>	<i>In vivo</i>
Apoptosis	Chromatin condensation; nuclear fragmentation; DNA fragmentation	Blebbing	Maintenance of organelle integrity; formation of apoptotic bodies	Caspase-dependent; release of apoptotic mediators from mitochondria	TUNEL assay; nuclear staining; DNA laddering; subG1/G0; annexin V; caspase-activity; mitochondrial changes	^{99m} Tc-annexin V scintigraphy; nonpeptidyl caspase binding radioligand?; ^{99m} Tc-MIBI?
Necrosis	Clumping and random degradation of nuclear DNA	Swelling; rupture; loss of ion pump/channel function	Increase vacuolation; organelle degradation; mitochondrial swelling	NAD/ATP depletion	Typical morphological changes	^{99m} Tc-annexin V scintigraphy; FDG/FLT-PET?
Mitotic catastrophe	Multiple micronuclei; nuclear fragmentation	–	–	Caspase-independent; altered gene expression	Aberrant mitosis; mitotic markers; TUNEL	–
Autophagy	Partial chromatin condensation	Blebbing	Autophagic vesicles	Activation of autophagy-related genes	Transmission EM; LC3; ubiquitin; flow cytometry	–
Senescence	Chromatin changes	–	Increased granularity	SA-beta-gal	EM; typical morphology; growth arrest assay	FDG/FLT-PET?

components brings about the typical apoptotic morphology. Inducer caspases are activated by multimerization on a scaffold protein, whereupon induced proximity allows for cross-cleavage and activation. Two major pathways for inducer caspase activation can be distinguished: the intrinsic pathway in which caspase-9 multimerizes at the Apaf-1 scaffold downstream from mitochondria, and the extrinsic pathway in which caspase-8 or caspase-10 multimerize at the cytoplasmic tail of death receptors [3].

Most apoptotic stimuli, including radiation depend on the intrinsic mitochondrial pathway in which induced permeability of the mitochondrial outer membrane permits the release of pro-apoptotic factors into the cytosol. Of these, cytochrome c binds to the scaffold protein Apaf-1 and allows recruitment and activation of the inducer caspase-9, forming the apoptosome complex. In addition, Smac/Diablo alleviates blockade of caspase-9 and effector caspases by the inhibitor of apoptosis proteins. The mitochondria also release other factors that contribute to morphological changes [4].

The Bcl-2 protein family mediates mitochondrial permeability and is therefore the key regulator of most apoptotic pathways [5]. The presence of Bcl-2 homology (BH) domains is their common characteristic. The family consists of the anti-apoptotic Bcl-2 group, the pro-apoptotic Bax group and the pro-apoptotic BH3 domain-only group.

Family members can heterodimerize through interaction of the BH3 domain α helix with a groove formed by BH1 and BH2 domains. Death-induction involves collaboration between Bax type proteins and BH3 domain-only family members [6]. The BH3 domain-only group has many members that differ in responsiveness to stimuli and in the pathways they regulate. They are controlled at the transcriptional and/or post-translational level [5]. For instance, Noxa and Puma are transcriptionally upregulated by p53 and implicated in DNA damage pathways, Bmf binds to the cytoskeleton and is activated upon cell detachment, Bad is sequestered in the cytosol and released upon dephosphorylation induced by growth factor deprivation, while Bid is activated by proteolysis, downstream from death receptors. The Bax group contains the ubiquitous Bax and Bak and the ovary-specific Bok. Of these, Bak is constitutively associated with mitochondria. Studies on Bax and Bak knock out cells have recently illuminated how pro-apoptotic Bcl-2 family members operate [6, 7]. BH3 domain-only proteins require at least one Bax type partner to induce cell death. They induce a conformational change in Bax/Bak and its assembly in the mitochondrial membrane into homomultimers with presumed channel forming properties [7–9]. Inhibitory Bcl-2 family members such as Bcl-2 and Bcl-X_L sequester BH3-domain only proteins, disallowing them to anticipate in pro-apoptotic

complex formation [10]. A refinement of this model suggests that some BH3 domain-only proteins specialize in complexing with inhibitory Bcl-2 family members, while others preferentially interact with and activate Bax or Bak [11].

Unlike all other apoptotic stimuli, death receptors can activate effector caspases and induce apoptosis independent of the mitochondria via the extrinsic pathway. Death receptors are members of the tumor necrosis factor (TNF) receptor superfamily and activate inducer caspase-8/10 at the death domain in their cytoplasmic tail. TNF-related apoptosis-inducing ligand (TRAIL) receptors and CD95 do this by a common principle. Upon ligand binding, preformed receptor trimers recruit Fas-associated protein with death domain (FADD), which in turn recruits caspase-8/10. Within this death-inducing signaling complex (DISC), caspase-8/10 are activated by multimerization. They are subsequently released into the cytosol where they cleave and activate effector caspases. Death receptors also connect to the mitochondrial pathway for caspase activation, which is regarded as an amplification loop for effector caspase activation. Upon cleavage by caspase-8/10, the carboxy-terminal fragment of BH3-only protein Bid (tBid) relocates to mitochondria where it induces membrane permeability via Bax or Bak. This releases mediators such as cytochrome c and Smac/Diablo that allow activation of caspase-9 and effector caspases.

Although radiation-induced apoptosis is caspase-dependent [12–14], it is not well understood how radiation activates the caspase cascade. The observation that Bcl-2 is able to block radiation-induced cytochrome c release and apoptosis [14–16], demonstrates the involvement of a mitochondria-dependent mechanism in this response. This notion is further supported by the fact that p53, which is activated by DNA-damaging agents like ionizing radiation, is a direct transcriptional regulator of both Bcl-2, Bax, Puma, Noxa and Bid [17–19] and can act as an apoptogenic factor at the mitochondrial membrane [20]. There are also other direct links between p53 and death receptors: the CD95 and TRAIL receptor-2 (DR5) genes contain a p53 response element and both receptors (and/or their respective ligands) can be upregulated in response to radiation and chemotherapeutic drugs [21–23], causing activation of the initiator caspase 8.

1.1.2 Non-apoptotic cell death

Non-apoptotic cell death includes necrosis, mitotic cell death (mitotic catastrophe), autophagy and senescence. These will be shortly discussed (Table 1).

Necrosis Necrosis is usually the cellular response to severe and massive toxic insults associated with infection, inflam-

mation or ischemia, such as defective membrane potential, cellular energy depletion, nutrient starvation, damage to membrane lipids or loss of function of ion channels/pumps. A major difference with apoptotic cell death which is energy-dependent, is the depletion of the adenosine triphosphate pool and production associated with necrosis. Conditions of increased intracellular calcium or nicotinamide adenine dinucleotide depletion by excessive activation of Poly adenosine diphosphate-ribose polymerase following DNA damage are believed to be a cause of necrotic cell death as well [24]. Necrotic morphology is characterized by increased cytoplasmic vacuolation, organelle degeneration, damage to membrane lipids with cell swelling and rupture, and induction of inflammation due to the release of cellular contents. Although necrosis is usually considered a passive and uncontrolled process, recent data indicate that this mode of cell death is partially under cellular control [25].

Mitotic catastrophe Mitotic catastrophe refers to the process when cells attempt to divide without proper repair of DNA damage. This aberrant mitosis is associated with giant, multinucleated cells and uncondensed chromosomes, distinguishing mitotic catastrophe morphologically from other types of cell death [26]. It is the most prominent mode of cell death after radiation in solid tumors and may occur at long intervals after exposure depending on the proliferation rate and DNA repair capacity of the irradiated cells [27, 28]. An essential element of the cellular response to radiation involves cell cycle arrest allowing the damaged DNA to be repaired before replication and mitosis occur [29]. When the damage is too extensive and repair fails, cells may undergo apoptosis. Frequently, however, these checkpoints are defective and cells may enter mitosis prematurely before DNA repair is completed. Because in a large proportion of tumors, cell cycle checkpoints are compromised and apoptotic pathways frequently suppressed, tumor cells preferentially execute this mitotic mode of cell death after treatment with DNA damaging regimens.

Autophagy Autophagy is an evolutionary conserved physiological mechanism responsible for the routine degradation and turnover of (non-essential) cytoplasmic components by lysosomal hydrolases [30]. Autophagy can be activated under various stress conditions such as starvation, hypoxia, hyperthermia and oxidative stress. In response to radiation autophagy has been shown to be associated with the development of acidic vesicular organelles [31]. Furthermore, activation of autophagy by inhibiting mammalian target of rapamycin signaling may contribute to the cellular response to radiation [32]. Defective autophagy has been implicated in a variety of diseases including vacuolar

myopathies, neurodegenerative disorders and cancer [33, 34]. Morphologically, autophagy is characterized by the appearance of a large number of cytosolic vacuole-like structures (autophagic vesicles). It remains unclear how autophagy is involved in tumor cell growth and whether inhibition of this mode of cell death modulates the response to anticancer treatment. Cross-talk between autophagic cell death and apoptosis has been described at the level of mitochondria, estrogen receptor and lysosomes [35].

Senescence Senescence is usually defined as a state of permanent loss of proliferative capacity. Two types of senescence have been described: replicative senescence which is associated with aging and results from a progressive shortening of telomeres during cell division, and accelerated senescence which can be induced by DNA damage, oncogenic mutation and treatment with telomere-shortening agents [36]. Because senescent cells have undergone irreversible growth arrest and lost clonogenic potential, they may constitute an important component of the tumor response to therapy [37]. The induction of senescence is accompanied by specific cellular morphological changes such as increased granularity in enlarged and flattened cells. A number of genes involved in cell cycle regulation have also been implicated in senescence, including p53, p21, Rb and p16.

1.2 Markers and monitoring of cell death

1.2.1 Apoptosis

For quantitative *in vitro* analysis of apoptosis, a number of assays are currently available. These include nuclear staining and cytometric DNA-fragmentation assays, terminal uridine deoxynucleotidyl transferase nick end labeling (TUNEL) staining, DNA laddering on sodium dodecyl-polyacrylamide gel electrophoresis, detection of subG1/G0 cell population, Annexin V staining, caspase-activity assays

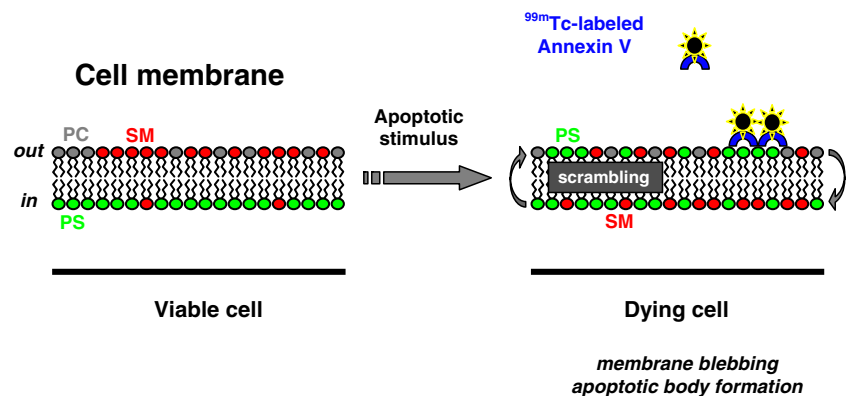
and detection of changes in mitochondrial membrane potential (Table 1). Some of these assays can be used for *ex vivo* detection of apoptosis in tumor tissue biopsies before, during and after treatment. Many studies have indeed been undertaken to establish a correlation between the level of apoptosis prior to, and the tumor response following irradiation. The results of these studies, however, have been conflicting. While some studies have shown that a high apoptotic index correlates with good prognosis, others show the reverse [38]. Both methodological differences and intra-/inter-tumor variations among these studies complicate interpretation of the results [39]. Moreover, since apoptosis is controlled by a large number of positive and negative regulators, focusing on the apoptotic index alone will certainly not lead to the identification of a solid predictor for treatment outcome. For example, the combination of mutated p53 and overexpression of Bcl-2 was identified as a highly significant unfavorable prognostic factor for local control and disease-free, disease-specific and overall survival in 83 patients with transitional cell cancer of the bladder treated by radiotherapy [40]. Thus, in addition to classical prognostic factors, apoptosis may be helpful in predicting tumor response to therapy. Obviously, larger and prospective randomized studies are needed to establish the role of such tumor biological parameters in predicting the natural history of a tumor and its radiosensitivity.

1.2.2 *In vivo* imaging of apoptosis

Our increased knowledge on the processes that regulate apoptosis has identified several targets that can be used as specific cell death markers, including membrane alterations, mitochondrial permeabilization and cytochrome c release, caspase activation, DNA degradation and nuclear fragmentation. One of the early events during the apoptotic program is the externalization of phosphatidyl serine (PS) from the cytoplasmic to the extracellular leaflet of the plasma membrane lipid bilayer (Fig. 1). This redistribution of PS

Fig. 1 Externalization of phosphatidyl serine allows binding of ^{99m}Tc -annexin V early during apoptosis.

PS = phosphatidyl serine;
SM = sphingomyelin;
PC = phosphatidyl choline



results from coordinated translocase, floppase and scramblase activities, and precedes most of the other events of the apoptotic cascade. When exposed PS serves as a binding site for Annexin V.

Annexin V is a member of a larger family of at least 13 calcium and phospholipid binding proteins [41]. It is widely expressed in eukaryotic cells where it is mainly confined to the cytosolic side of the plasma membrane. In addition, Annexin V can be found at low plasma concentrations in healthy humans [42]. The protein consists of 319 amino acids forming a single planar cyclic polypeptide chain with a total molecular weight of 35.8 kDa. Originally, Annexin V was implicated in blood coagulation as it inhibits prothrombin activation and prevents thrombus formation. It also displays anti-inflammatory properties through its inhibitory effect on phospholipase A2 activation, preventing the release of arachidonic acid [43]. Finally, Annexin V has been shown to act as an inhibitor of protein kinase C. Most of these biological functions of Annexin V can be explained by its high calcium-dependent binding affinity for negatively charged phospholipids, in particular for PS with nanomolar affinity [44]. Normal, quiescent cells can harbor up to 5,000 PS-binding sites for Annexin V [45], while this number can increase 1,000-fold in activated thrombocytes and endothelial cells. Importantly, in apoptotic tumor cells as many as $6\text{--}14 \times 10^3$ /cell PS-binding sites can be found [46].

Based on these observations, Annexin V is regarded as a useful marker for PS-exposing cells when labeled with detectable tags. Indeed, fluorescent residues like fluorescein isothiocyanate are now routinely used for cell-sorting purposes by fluorescence-activated cell sorting and cytological and histological identification of apoptotic cells.

More recently, *in vivo* detection of apoptosis has become feasible with the development of radiolabeled Annexin V-based PS-targeting molecules. Recombinant Annexin V coupled to the bifunctional chelator molecule hydrazinonicotinamide (HYNIC) and radiolabeled with ^{99m}Tc is currently the most widely studied and extensively used tracer for *in vivo* visualization and quantification of apoptosis. Initial studies focused on a variety of experimental models, including CD95/Fas-induced hepatic apoptosis [47]. Anti-CD95/Fas antibody-treated mice demonstrated a three-fold rise in hepatic uptake of Annexin V above control, identified both by imaging and scintillation well counting. The increase in hepatic uptake in anti-CD95/Fas antibody-treated mice correlated to histological evidence of fulminant hepatic apoptosis. In patients, ^{99m}Tc -HYNIC-Annexin V has successfully been applied in studies on myocardial ischemia [48], transplantation medicine [49] and hypoxic brain injury [50]. In oncology, ^{99m}Tc -HYNIC-Annexin V is increasingly being employed to monitor and predict the response to anti-cancer therapy. In one of the first clinical

studies the safety and feasibility of ^{99m}Tc -Annexin V (TAV) for imaging chemotherapy-induced apoptosis in various malignancies immediately after the first course of chemotherapy was evaluated [51]. Fifteen patients presenting with lung cancer, lymphoma or breast cancer underwent ^{99m}Tc -Annexin V scintigraphy before and within 3 days after their first course of chemotherapy. Tumor response was evaluated by computed tomography (CT) and positron emission tomography (PET) scans, 3 months after completing treatment. In all cases, no tracer uptake was observed before treatment. However, 24–48 h after the first course of chemotherapy, seven patients who showed ^{99m}Tc -Annexin V uptake at tumor sites, suggesting apoptosis, had a complete or partial response. Conversely, six of the eight patients who showed no significant post-treatment tumor uptake exhibited progressive disease. Despite the lack of tracer uptake after treatment, the two patients with breast cancer had a partial response. Overall survival and progression-free survival were significantly related to tracer uptake in treated lung cancers and lymphomas. These data suggest that early ^{99m}Tc -Annexin V tumor uptake may be a predictor of response to treatment in patients with late stage lung cancer and lymphoma.

It should be noted, however, that (tumor) cells dying by necrosis also bind Annexin V. This is explained by the fact that PS in the inner leaflet becomes accessible for Annexin V binding as soon as the plasma membrane is ruptured and becomes permeable during necrosis. This limits the specificity of PS targeting by Annexin V ligands and hampers the *in vivo* discrimination between apoptotic and necrotic cell death. Therefore, Haas et al. [52] focused on a tumor entity known to be extremely radio- and chemosensitive and to respond by extensive induction of apoptosis [53, 54]. In a series of 11 low grade follicular lymphoma patients ^{99m}Tc -Annexin V scintigraphy (TAVS) was performed before and 24 h after the last fraction of 2×2 Gy involved field radiotherapy. Fine-needle aspiration cytology of the target lymphoma was performed on five consecutive days to verify the apoptotic nature of the response. Baseline tracer uptake was absent in six and weak in five patients. Optimal timing for cytological apoptosis assessment was found to be between 24–48 h after treatment. In all but one patient the post-treatment TAV uptake matched the post-treatment cytology and correlated with the type and onset of the clinical response (two partial and eight complete remissions). These results indicate that the increase in TAV uptake after low dose radiotherapy correlated with the induction of apoptosis and predicted outcome. In a subsequent study of 38 patients with different tumor types (non-Hodgkin lymphoma (NHL) $n=31$; non-small cell lung carcinoma (NSCLC) $n=4$; head and neck squamous cell carcinomas $n=3$) treated with various modalities (radiotherapy $n=26$; cisplatin-based chemotherapy

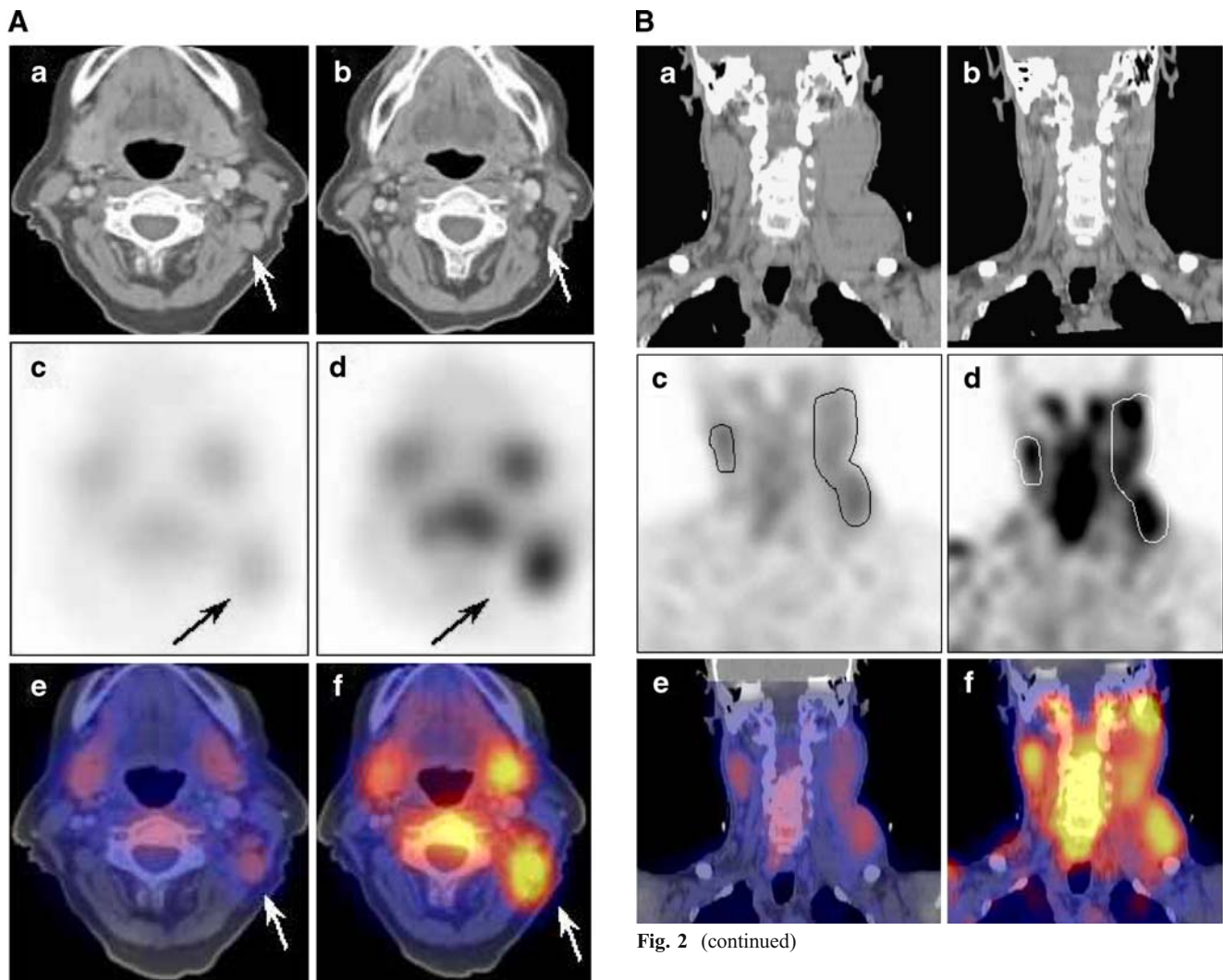


Fig. 2 (continued)

Fig. 2 **A** *In vivo* ^{99m}Tc -annexin V scintigraphy. An example of baseline (a, c, e) and 24 h post-treatment scans (d, f). Diagnostic CT (a, b) of a patient with NHL is shown. This patient demonstrated a complete response after low dose (2×2 Gy) radiotherapy on follow-up CT scan (b). TAVS (c, d) and SPECT/CT fusion images (e, f) show precise localization of increased tracer uptake. **B** *In vivo* ^{99m}Tc -annexin V scintigraphy. An example of baseline (a, c, e) and 72 h post-treatment scans (d, f). Diagnostic CT (a, b) of a patient with HNSCC is shown. This patient demonstrated a complete response after cisplatin-based chemo-radiotherapy on follow-up CT scan (b). TAVS (c, d) and SPECT/CT fusion images (e, f) show precise localization of increased tracer uptake. **C** *In vivo* ^{99m}Tc -annexin V scintigraphy. An example of a baseline (a, c, e) and 48 h post-treatment scan (d, f). Diagnostic CT (a, b) of a patient with NSCLC is shown. This patient demonstrated a partial response after cisplatin-based chemotherapy on follow-up CT scan (b). TAVS (c, d) and SPECT/CT fusion images (e, f) show precise localization of increased tracer uptake. Figure 2A is reprinted from: *Radiotherapy and Oncology* 72(3), 333-339 (2004) Kartachova M, Haas RLM, Valdés Olmos RA, Hoebbers FJP, van Zandwijk N, Verheij M. *In vivo* imaging of apoptosis by ^{99m}Tc -Annexin V scintigraphy: visual analysis in relation to treatment response, with permission from Elsevier. Figures 2B and 2C are reprinted from: *Current Medical Imaging Reviews* 1, 221-228 (2005) Kartachova M, Verheij M, van Eck B, Hoefnagel K, Valdés Olmos R. *Methodological aspects and applications of in vivo imaging of apoptosis in oncology: an illustrative review*, with permission from Bentham Science Publishers Ltd

$n=4$; cisplatin-based concurrent chemoradiotherapy $n=3$), TAVS was acquired before and within 48 h after the start of anti-cancer therapy [55, 56]. The changes in TAV tumor uptake (ΔU) were visually and quantitatively evaluated and compared with response to treatment according to Response Evaluation Criteria In Solid Tumors (RECIST) criteria (Fig. 2). A statistically highly significant correlation ($p < 0.0001$) was found between ΔU and treatment outcome for both visual and quantitative analysis (see updated analysis in Fig. 3). Recently, the prognostic significance of TAVS during chemotherapy in lung cancer was evaluated. In 16 consecutive chemotherapy-naïve patients with advanced stage NSCLC scheduled for cisplatin-based chemotherapy, TAVS was performed before and within 48 h after the start of therapy. Chemotherapy-induced changes in tumor Annexin V uptake, calculated as maximum count per pixel and expressed as percentage to baseline value, were compared with treatment response determined according to RECIST. A significant correlation ($r^2=0.86$; $p=0.0001$) was found between Annexin V metabolic changes and

Fig. 2 (continued)

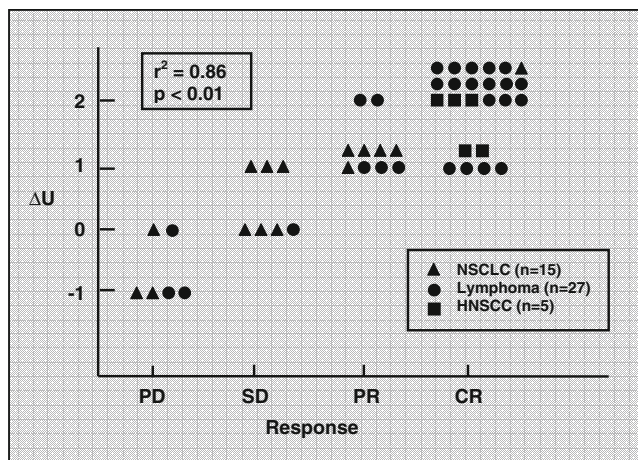
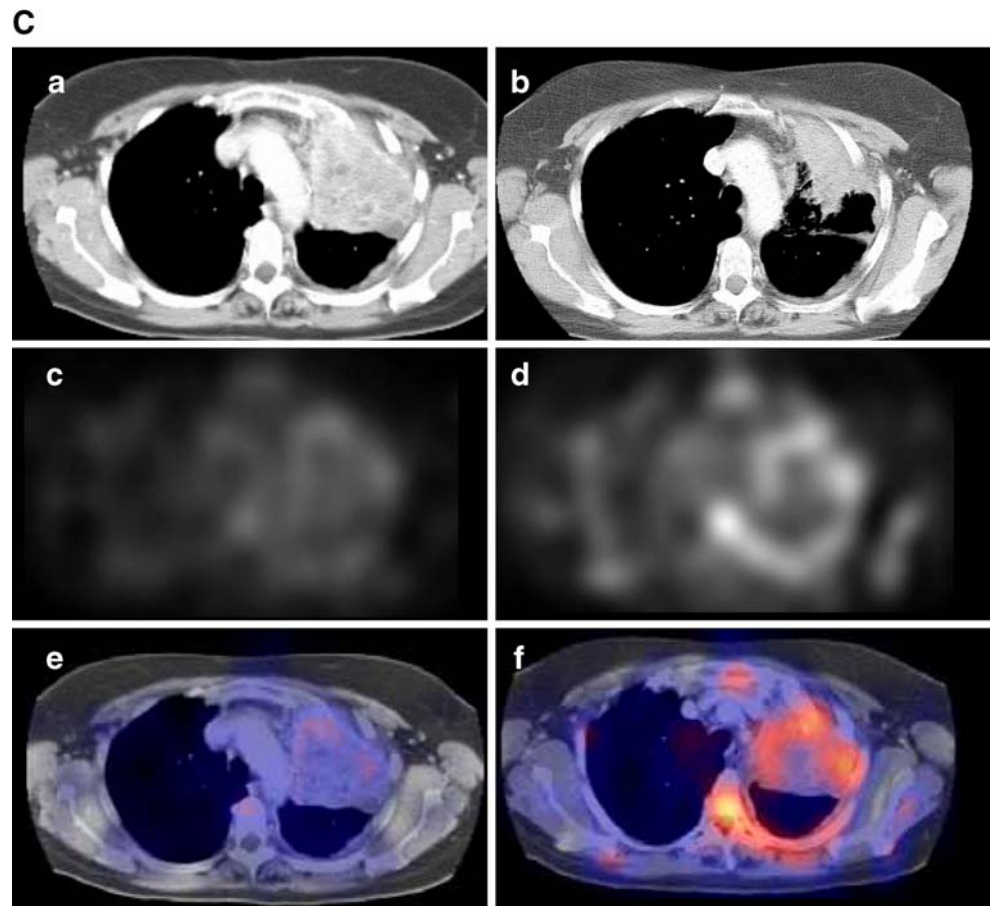


Fig. 3 Schematic correlation between changes in TAV tumor uptake (ΔU) and clinical response after treatment for NSCLC ($n=15$; cisplatin-based chemotherapy), NHL ($n=27$; low dose 2×2 Gy radiotherapy) or HNSCC ($n=5$; cisplatin-based chemoradiotherapy). ΔU was determined according to a semi-quantitative grading scale. Spearman rank test showed a significant correlation. *PD* = progressive disease; *SD* = stable disease; *PR* = partial response; *CR* = complete response; see also [52, 55, 56]

treatment outcome. All patients with notably increased Annexin V tumor uptake showed complete or partial response. Less prominently increased or decreased uptake correlated with stable or progressive disease.

Collectively, these studies indicate that TAVS might be useful as a predictive test for treatment response early during the course of treatment. It may therefore offer the possibility to identify those patients who will respond to treatment avoiding toxic and ineffective regimens for those patients who will resist therapy. Despite these promising results, the evaluation of TAVS data is complicated by a low tumor-to-background ratio and the limited spatial resolution of the gamma camera. To overcome these limitations, co-registration of functional (single-photon emission computed tomography; SPECT) and anatomical information (CT, MRI) can be applied [57, 58] (Fig. 2). This provides a detailed localization of the areas of increased tracer uptake within anatomical structures, and allows not only to compare tumor uptake on pre- and post-treatment scans on corresponding levels, but also to discriminate areas of pathological tracer accumulation from

structures with enhanced physiological uptake, such as the bone marrow and salivary glands.

In addition to Annexin V scintigraphy, other approaches for *in vivo* imaging of apoptotic cell death are being developed. Perhaps the most promising strategy involves targeting of activated caspases because of the higher degree of specificity as compared to the PS-targeting approach of Annexin V which also detects necrotic cell death [59]. Finally, the use of technetium-99m methoxyisobutylisonitrile (^{99m}Tc -MIBI) is worth mentioning because this tracer accumulates within mitochondria depending on the Bcl-2 status. In several studies it has been shown that absent or reduced early ^{99m}Tc -MIBI uptake in tumors is associated with a Bcl-2-mediated resistance to chemo- and radiotherapy [60].

1.2.3 Necrosis

In tumor tissue samples necrotic cells are routinely identified by established morphological criteria. Particularly in hypoxic areas this type of cell death is abundantly present. No specific imaging modalities are available for *in vivo* monitoring of necrosis. As mentioned above, Annexin V scintigraphy does not discriminate between apoptotic and necrotic cell death. Fluorodeoxyglucose-18 PET is increasingly used to monitor response to treatment in tumors [61], but tracer accumulation does not occur in necrotic areas.

1.2.4 Mitotic catastrophe

The formation of multinucleated cells indicative of mitotic catastrophe can be identified in tissue samples by standard techniques. No specific *in vitro* or *in vivo* assays are available for this mode of cell death.

1.2.5 Autophagy

The detection of autophagy is limited to *in vitro* and *ex vivo* settings and mainly relies on morphological assays to detect autophagic vesicles. Transmission electron microscopy is currently the standard method to monitor autophagy in tissue. In addition, immunohistochemical staining of autophagy-specific biomarkers such as microtubule-associated protein 1 light chain 3 appeared to be a valuable technique to detect autophagosome formation in tissue. Furthermore, demonstration of granular cytoplasmic ubiquitin inclusions by immunohistochemistry may be an attractive technique to measure autophagic cell death in some human diseases such as neurodegenerative disorders, heart failure and atherosclerosis [62]. Other approaches developed to monitor autophagy include flow cytometric analysis of acridine orange uptake and measurement of cytosolic lactate dehydrogenase contents. To date there are no imaging modalities to monitor autophagy *in vivo*.

1.2.6 Senescence

Senescent cells in culture are identified by their inability to undergo DNA synthesis. Biochemically, senescence is accompanied by the induction of senescence-associated β -galactosidase (SA-beta-gal), which can be detected by histochemical staining of cells using the artificial substrate X-gal. [63]. The presence of the SA-beta-gal biomarker is independent of DNA synthesis and generally distinguishes senescent cells from quiescent cells. The method to detect SA-beta-gal is single cell-based assay, which can identify senescent cells even in heterogeneous tumor cell populations. *In vivo*, the permanent loss of proliferative capacity may be derived from changes in PET imaging with ^{18}F -FDG or ^{18}F -FLT during treatment.

2 Conclusions

The cellular response to radiation is complex and may lead to different biological outcomes. Understanding the underlying molecular mechanisms of the various types of cell death is essential for the identification of useful biomarkers and the development of imaging modalities to monitor and predict tumor response to radiation. This becomes even more important with the recent introduction of biological response modifiers that target the radiation response at different levels. ^{99m}Tc -Annexin V scintigraphy represents an apposite example of a non-invasive technique that allows visualization and quantification of radiation induced cell death *in vivo*. Clearly, additional and more specific tests are needed for accurate patient selection to ensure optimal treatment and limit side effects.

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