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DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling

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Abstract

Genotoxic DNA damaging agents may activate both membrane death receptors and the endogenous mitochondrial damage pathway leading to cell death *via* apoptosis. Here, apoptotic responses in cells exhibiting a defect in various DNA repair pathways such as alkyltransferase, base excision repair, nucleotide excision repair and mismatch repair are reviewed. The HSVTk/ganciclovir and VZV/BVDU suicide system will also be discussed. Data are available to show that critical DNA damage triggers apoptosis in a DNA replication dependent way by activating the mitochondrial damage pathway in fibroblasts. It is proposed that DNA double-strand breaks (DSBs) are common ultimate apoptosis-triggering lesions arising from primary DNA lesions during DNA replication. Thus, DNA replication is a necessary component in DNA damage-triggered apoptosis, at least in fibroblasts treated with genotoxins not inducing DSBs themselves. For methylating agents inducing O^6-methylguanine, an additional requirement is mismatch repair provoking DSB formation that triggers Bcl-2 decline and caspase-9/-3 activation. This occurs independent of p53 since most of the repair deficient cell lines under study were mutated for p53. Moreover, p53 knockout fibroblasts are more sensitive to methylating agents and UV light than p53 wt cells, suggesting p53 to play a protective rather than a pro-apoptotic role in this cell system, probably by its involvement in DNA repair. However, for lymphoblastoid cells p53 wt variants are more sensitive to DNA damage indicating that p53 participates in apoptotic signaling in a cell type-specific fashion. The role of topoisomerase II inhibitors and c-Fos/AP-1 in apoptosis will also be discussed.

Keywords: Apoptosis; DNA damage; DNA repair; Cell cycle; Cell death; Tumortherapeutic agents

1. Introduction

There is general consensus that DNA is the main target for most, if not all genotoxic agents. These include environmental alkylating carcinogens, ultraviolet (UV)-light and ionizing radiation (IR) as well as a large amount of anticancer drugs. If not repaired prior to replication, DNA damage is generally harmful resulting in effects detectable on the level of chromosomal aberrations, recombinations (SCEs) as well as gene mutations and malignant transformation (Fig. 1). For these genotoxic endpoints, the critical lesions and the molecular mechanisms involved have been extensively studied over decades leading to a growing understanding of the mutagenic and genotoxic responses of cells [1]. For the endpoint cytotoxicity, however, our knowledge seems to be quite fragmented, since cells die because of a complex intrinsic mechanism that becomes activated upon genotoxic exposures. The main route of genotoxic killing appears to be programmed cell death (apoptosis). Since apoptosis can be induced by a large number of physiological stimuli targeting membrane receptors, critical targets other than DNA must also be considered in genotoxin-induced apoptosis. In this brief report data obtained in the author's laboratory will be summarized to show that DNA damage (but not DNA damage independent receptor activation) is the major cause of genotoxin-induced apoptosis in fibroblasts treated with various genotoxic agents, including anticancer drugs. It is also shown that apoptosis in fibroblasts is executed by

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Abbreviations: SCEs, sister chromatid exchanges; UV, ultraviolet; IR, ionising radiation; MGMT, O^6-methylguanine-DNA methyltransferase; MMR, mismatch repair; BER, base excision repair; NER, nucleotide excision repair; TCR, transcription coupled repair; GCV, ganciclovir; PCV, penciclovir; BVDU, bromovinyldeoxyuridine; HSVTk, herpes simplex virus thymidine kinase; VZVTk, varizella zoster virus thymidine kinase; HSV-*tk*, herpes simplex virus thymidine kinase gene; O^6MeG, O^6-methylguanine; SSBs, single strand breaks; DSBs, double strand breaks; Polβ, DNA polymerase β; p53 ko, p53 knockout.

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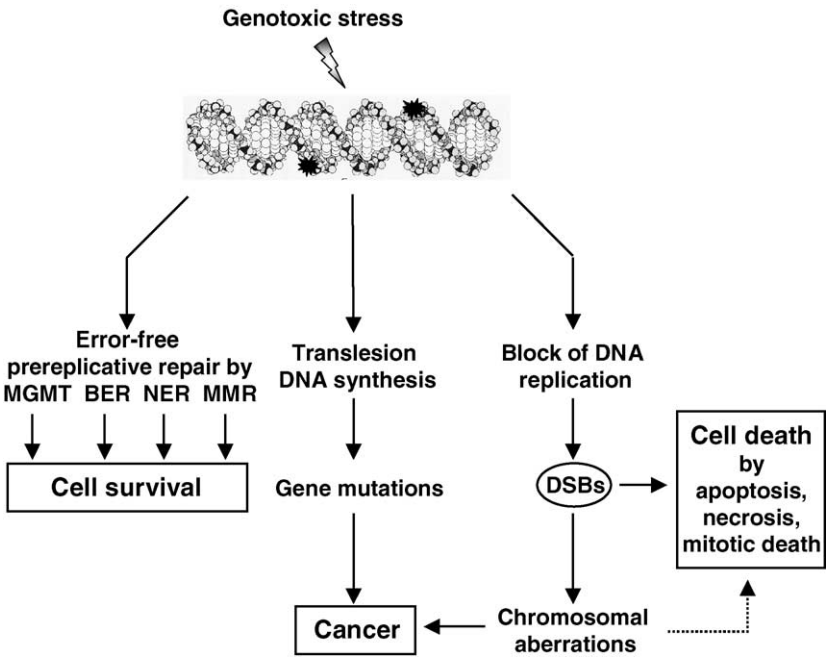

Fig. 1. DNA damage-induced endpoints. DNA lesions are subject to pre-replicative repair by MGMT, BER, NER and MMR allowing cells to survive and preventing mutations. Mispairing and replication blocking lesions cause gene mutations because of misincorporation of bases during translesion DNA synthesis. Bulky DNA lesions may also cause replication arrest provoking nuclease attack at stalled replication forks leading to DSB formation which was found to correlate with chromosomal aberrations and cell killing [23,25,40].

activating the mitochondrial damage pathway. Finally the role of p53 and Fos/AP-1 will briefly be discussed.

2. Experimental systems

Probably the best way to determine how DNA damage is involved in triggering apoptosis is the utilization of DNA repair deficient cell lines that are characterized by their inability to remove a particular type(s) of DNA damage. Alternatively, DNA can be specifically damaged by incorporation of a specific modified DNA precursor. Consequently, the following experimental systems have been utilized and will be discussed: (a) O^6-methylguanine-DNA methyltransferase (MGMT) deficiency together with impaired mismatch repair (MMR); (b) base excision repair (BER) deficiency; (c) nucleotide excision repair (NER) deficiency; and (d) ganciclovir (GCV) incorporated into DNA of herpes simplex virus-thymidine kinase (HSVTk) expressing cells. Moreover, the effect of penciclovir on VZVTk cells and the response of p53 and c-Fos knockout cells will be summarized.

3. Apoptosis in MGMT deficient, MMR proficient cells

MGMT is a key suicide enzyme repairing the promutagenic and procarcinogenic methylation lesion O^6-methylguanine (O^6MeG) in DNA. Lack of MGMT results in a methylation-hypersensitive phenotype characterized by increased induction of apoptosis [2]. This requires MMR; cells not expressing MMR proteins such as MSH2 or MSH6 are methylation-tolerant [3]. Based on this, compelling evidence was provided that the minor alkylation lesion O^6MeG (amounting only to <8% of total DNA methylations) is the major lesion responsible for cell death by apoptosis [2,4–6]. The current model proposed on the basis of available data suggests that O^6MeG in DNA is converted during the first DNA replication cycle into O^6MeG/T mispairs, which are subject to MMR. Here a dimer composed of MSH2/MSH6 is critically involved [5], forming the MutSα complex, which binds to the lesion and initiates its repair. During erroneous processing of O^6MeG/T mispairs (because the mispairing lesion O^6MeG provokes a faulty MMR cycle) critical secondary lesions are formed which are supposed to interfere with the subsequent round of DNA replication giving ultimately rise to DNA DSBs (Fig. 2). DSBs have been experimentally shown to be generated in MGMT−/MMR+ cells; these breaks preceded apoptosis [7]. A hallmark of O^6MeG-triggered apoptosis in fibroblasts is the decline of Bcl-2, which occurs dose-dependently prior to the onset of apoptosis. Transfection with *bcl-2* protected, at least to some extent, against O^6MeG-triggered apoptosis [7]. We also observed release of cytochrome *c*, caspase-9/-3 activation and PARP-1 cleavage. We did not observe an increase in Fas receptor or Fas ligand expression or significant caspase-8 activation [7]. Cells included in this study were p53 mutated, suggesting O^6MeG to activate the mitochondrial damage pathway in a p53-independent way.

The model proposed for O^6MeG-triggered apoptosis implicates the requirement of DNA replication and the

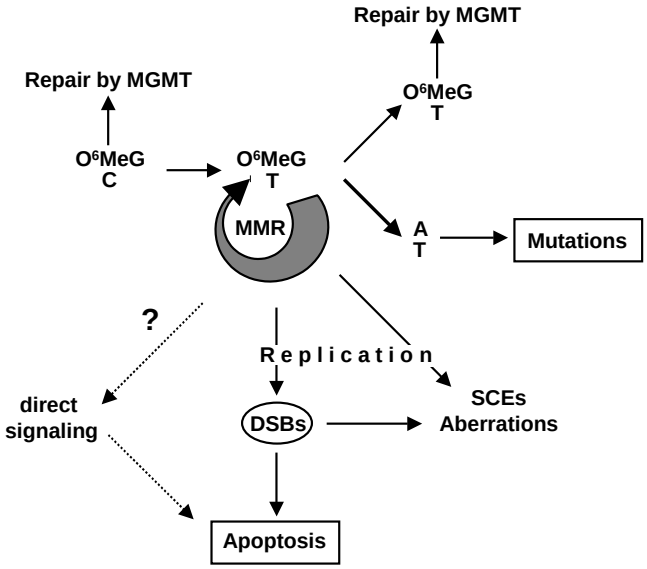

Fig. 2. O^6-MeG-triggered genotoxic pathways. O^6MeG can be repaired by MGMT prereplicatively or postreplicatively upon pairing with thymine [41]. Mispairing of O^6MeG with thymine is the major source of GC → AT transition mutations induced by methylating carcinogens. O^6MeG/T mispairs are subject to erroneous MMR giving rise to secondary lesions that interfere with DNA replication. This leads to the formation of SCEs, chromosomal aberrations and DSBs which finally trigger apoptosis.

formation of DSBs. Both have been experimentally shown to be true. Thus, CHO fibroblasts cultivated under serum starved conditions do not display significant apoptosis.[1] Also, O^6-methylating agents induce apoptosis in human lymphocytes only if they were stimulated to divide [42]. The formation of DSBs has been shown in MGMT−/MMR+ cells (but not in MGMT+/MMR− or MGMT−/MMR− cells) which preceded apoptosis. Furthermore, apoptosis triggered by O^6MeG is a quite late response occurring 2–3 days after pulse-treatment with MNNG, indicating that it occurs not during the treatment but in the first or second post-treatment cell cycle [7].

It has repeatedly been discussed that the MutSα complex, which is able to bind to both O^6MeG/C and O^6MeG/T lesions, may directly provide a signal downstream to the apoptotic machinery; thus it could be considered as a "sensor" of apoptotic DNA lesions (see Fig. 2). This model, however, did not gain experimental verification. The late apoptotic response (in the second and third cell cycle after alkylation) as well as the requirement of DNA replication are clear arguments disproving the "sensor" model. Moreover, MutSα also binds to UV-C lesions and several other bulky DNA adducts [8] but the apoptotic effect is specific for O^6-methylating agents. Thus, MGMT−/MMR+ (tolerant) cells are no more resistant to UV-C light than MGMT−/MMR− cells[2] which one would not suppose on the basis of the "sensor" model.

[1] Dunkern and Kaina, unpublished data.
[2] Unpublished data.

4. Apoptosis in BER mutants

BER removes *N*-alkylation lesions such as *N*7-methylguanine, *N*3-methyladenine and *N*3-methylguanine from DNA. Cells deficient in DNA polymerase β (Polβ) are hypersensitive to simple alkylating agents [9] responding with a high frequency of apoptosis [10]. Similar to O^6MeG-triggered apoptosis, Polβ deficient cells require DNA replication in order to become apoptotic upon methylation. Again, supported by experimental data, the formation of DSBs appears to be involved [11]. A model was proposed suggesting that DNA single-strand breaks (SSBs) are yielded at high frequency in Polβ knockout cells because of the inability of these cells to perform short patch BER to completion. Non-sealed SSBs will then be transformed into DSBs during S-phase, triggering Bcl-2 decline and the mitochondrial damage pathway [11].

5. Apoptosis in NER mutants

Chinese hamster cells deficient in NER are hypersensitive to UV-C. This hypersensitivity is mainly due to increased induction of apoptosis [12]. Apoptosis induced in NER defective Chinese hamster fibroblasts (ERCC1 and ERCC3 mutants, respectively) is a late response, very likely occurring predominantly in the second post-treatment cell cycle. This has been demonstrated by microscopic quantitation of apoptotic cells, which were simultaneously labeled by BrdU. About 70% of apoptotic cells were BrdU positive, indicating that these cells have passed through S-phase after UV irradiation. Using serum starvation and high density seeding protocols, replication dependence of UV-C induced apoptosis in NER fibroblasts was confirmed [13]. UV-C triggered Bcl-2 decline and caspase-9/-3 and -8 activation. Similar data was obtained with cisplatin in NER mutants, which also showed that non-repaired cisplatin DNA adducts are the primary trigger of cisplatin-induced apoptosis [14]. Caspase-8 activation upon UV-C irradiation was not the result of Fas activation, because transfection with TDN-Fas did not affect UV-C induced apoptosis in these cells. On the other hand, transfection with *bcl-2* provided protection against UV-C induced apoptosis, supporting the view that non-repaired UV-C lesions activate the mitochondrial damage pathway in a replication dependent manner by down regulation of Bcl-2 [12,13].

From work with Cockayne's syndrome cells exhibiting a defect in transcription coupled repair (TCR) it has been concluded that block of transcription may result in apoptosis [15]. Indeed, NER mutants display a stronger UV-induced block to transcription than wild-type cells. This transcription blockage could contribute to a minor fraction of apoptosis, which is replication independent (<30%) [13]. Interestingly, inhibition of transcription by actinomycin D was very efficient in provoking apoptosis, which was again characterized by a decline in Bcl-2. Cells

irradiated with UV-C display a high frequency of chromosomal breakage before they enter apoptosis, suggesting that DNA breakage (either DSBs *per se* or chromosomal aberrations) is again critically involved [13]. Similarly, the crosslinking agent glufosfamide, which is a glucose conjugate of ifosfamide mustard, induced apoptosis at high level in cells likely to be defective in crosslink repair. This was preceded by a wave of DSBs supporting the model that non-repaired crosslinks provoke the formation of DSBs during DNA replication, which finally trigger apoptosis [16]. Whether blockage of transcription is able to induce DSBs is an open question, which is currently under study.

6. Apoptosis in HSVTk expressing cells treated with GCV

Chinese hamster cells stably transfected with herpes simplex thymidine kinase gene (HSV-*tk*) are highly sensitive to the virostatic drug GCV, compared to non-transfected cells. This hypersensitivity is due to the induction of apoptosis; necrosis was only marginally induced [17]. GCV provoked genotoxicity in HSVTk cells at a high level as determined by a high yield of SCEs and chromosomal aberrations. The agent was shown to exert its genotoxic effect by incorporation into DNA. This happens specifically in HSVTk cells, which are able to metabolize GCV into the corresponding triphosphate. Neither SCEs nor chromosomal aberrations were formed in the incorporation cell cycle, whereas a high yield of genomic changes was brought about in the post-exposure replication cycle [17]. Similarly, apoptosis was induced not in the exposure but in the post-exposure cell cycle(s). This was accompanied by the occurrence of DSBs, Bcl-2 decline and caspase-9/-3 and -8 activation [18]. Whilst for alkylating agents Bcl-2 was degraded without the appearance of a Bcl-2 cleavage fragment, in the case of GCV-triggered apoptosis, a Bcl-2 fragment (p23) could be detected resulting very likely from caspase-9, which was shown to cleave hamster Bcl-2 protein [19]. This may provoke an amplification loop of mitochondrial damage-related apoptosis. Together with the fact that GCV is highly efficient in inducing DNA breakage, caspase provoked amplification of mitochondrial leakage might be a reason for the high ability of GCV to trigger apoptosis without significantly inducing necrosis. Whilst with CHO fibroblasts the apoptotic effect of GCV was receptor independent, death receptor activation was reported to be involved in neuroblastoma cells [20] indicating that the cellular background may impact GCV-triggered signaling.

From the fact that GCV becomes incorporated into DNA during replication, we inferred that GCV-triggered apoptosis is primarily due to DNA damage. This gained support by the finding that DNA polymerase β (Polβ) knockout cells are more sensitive to GCV than the corresponding wild-type. Also, prunasin—an inhibitor of Polβ—provoked GCV hypersensitivity [21]. This shows DNA repair is able to attenuate the killing effect of this antiviral drug.

7. Apoptosis in VZVTk expressing cells treated with bromovinyl deoxyuridine (BVDU)

Extending the approach of virostatic drug-induced apoptosis, we stably transfected CHO cells with the varizella zoster thymidine kinase (VZV-*tk*) gene, making the cells competent to metabolize the widely used antiviral drug BVDU. VZV-*tk* transfected cells became highly sensitive to BVDU, which was due to the induction of both necrosis and apoptosis. Interestingly, BVDU induced apoptosis was not related to Bcl-2, Bcl-X_L or Bax, which remained unchanged in expression. It was, however, related to a clear induction of Fas-L and caspase-8/-3 activation. BVDU induced c-Jun and, consequently, AP-1, which has been reported to upregulate the *fas-L* gene [22]. The response of VZVTk cells was completely different from that of HSVTk cells treated with GCV: BVDU stimulated the Fas pathway specifically, whereas GCV activated the endogenous mitochondrial pathway. Again, p53 (for which CHO cells are mutated) was not involved. As opposed to GCV, BVDU did not induce significant DNA breakage or clastogenic effects [23]. This might be a reason for the inefficiency of the agent to activate the mitochondrial damage pathway.

8. Apoptosis in cells upon DNA cleavage by restriction enzyme

Probably the most convincing way to demonstrate that DSBs trigger apoptosis is to induce DSBs selectively in DNA. This cannot be achieved by IR or chemical genotoxic exposures, which generate a broad spectrum of DNA lesions and, moreover, RNA, protein and membrane damage. It can be achieved, however, by means of electroporation of restriction enzymes into living cells, which proved to be a powerful method for inducing chromosomal damage [24]. We made use of this approach by introducing the blunt-end cutter *Pvu*II into mouse fibroblasts. *Pvu*II electroporation gave rise to a dose-dependent increase in DSBs peaked at 6 hr after electroporation. This was accompanied by a high yield of apoptosis, which appeared 2–3 days after treatment [25]. The reason for this long lag-phase is unclear. It might be due to inhibition of proliferation upon electroporation or long-lasting signaling mechanism. In parallel experiments, the same cells were treated with IR, which induced DSBs dose-dependently and, again at late times, apoptosis. Interestingly, IR induced both apoptosis and necrosis (as measured by annexin V/propidium iodide double staining and flow cytometry) at nearly equal proportions whereas *Pvu*II treatment provoked the induction of apoptosis only. Treatment with *Pvu*II and IR also caused decline in the level of

Bcl-2 [25]. These findings strongly support the view that DSBs are critical ultimate apoptotic lesions that are very efficient in triggering specifically the apoptotic mitochondria related pathway.

9. Apoptosis in p53 wt vs. p53 ko cells

The tumor suppressor protein p53 is considered to be a major player in the apoptotic response to genotoxins. Therefore we were interested in elucidating the role of p53 in the cell systems we were working on in more detail. The experiments on *Pvu*II electroporation summarized above were performed with mouse fibroblasts, which were either wild-type for p53 (p53 wt) or p53 deficient (derived from knockout mice, $p53^{-/-}$). Most interestingly, $p53^{-/-}$ fibroblasts were much more sensitive to *Pvu*II-induced apoptosis than p53 wt cells [24]. This indicates that p53 is not required for eliciting the apoptotic effect in fibroblasts. It rather exerts a protective effect. Similarly, p53 was not required for triggering apoptosis in DNA repair deficient CHO mutants which neither express p53 nor are able to transactivate the p53 promoter and are therefore considered to be mutated for p53 [14]. Nevertheless, they undergo apoptosis very efficiently, which obviously occurs in a p53 independent way.

To elucidate in more detail the role of p53 in DNA damage-triggered apoptosis, we compared p53 wt and $p53^{-/-}$ fibroblasts as to UV-C and alkylating agent (methyl methanesulfonate) induced apoptosis. Mouse fibroblasts deficient for p53 were clearly more sensitive than the corresponding wild-type. This has been proven both with primary [26] and established [27] cell lines, again supporting the view that p53 is not required for inducing apoptosis in fibroblasts. It rather protects against genotoxin-induced apoptosis in this cell system. This has been discussed to be due to direct involvement of p53 in DNA repair [28,29] and/or by p53-mediated blocking of G_1/S transition thus extending the time for prereplicative repair of DNA [30]. The pro- vs. antiapoptotic effect of p53 appears to be a cell type-specific phenomenon since lymphoblastoid cells wild-type for p53 proved to be more sensitive to alkylating agents and UV-C than the p53 mutated counterparts.[3] It will be a challenging task to identify, in future work, the factor(s) involved in making the decision between protection against or stimulation of the apoptotic process by p53.

10. Agent specificity in the induction of apoptosis: effect of topoisomerase inhibitors

Inhibitors of DNA topoisomerase II, such as doxorubicin and etoposide, were reported to be able to stimulate apoptosis by activating Fas receptor [31] or, alternatively (depending on the cell type), the mitochondrial damage pathway [32]. To study the role of p53 in this process, we investigated apoptosis induced by doxorubicin and etoposide in mouse fibroblasts that were wild-type or deficient for p53. To our surprise, in contrast to exposure to UV light and alkylating agents, p53 wt cells were clearly more sensitive to topoisomerase II inhibitors than $p53^{-/-}$ cells [33]. This was initially interpreted as p53 being directly involved in triggering apoptosis upon topoisomerase II inhibition. However, the cell lines did not differ in Fas expression upon doxorubicin exposure. On the other hand, upon doxorubicin and etoposide treatment p53 wt cells exhibited a significantly higher number of DNA strand breaks than $p53^{-/-}$ cells. This led us to propose that p53 is involved in generating DNA breaks rather than apoptotic signaling if topoisomerase inhibition occurs [33]. This hypothesis accounts for the fact that p53 physically interacts with DNA topoisomerase II [34] which might stimulate DNA incision or exaggerate the effect of doxorubicin and etoposide on the blockage of the religation function of the enzyme.

11. Role of Fos/AP-1 in DNA damage induced apoptosis

Cells deficient in c-Fos are hypersensitive to UV-C light [35] and a wide variety of DNA damaging agents including various anticancer drugs [36]. This hypersensitivity was shown to be due to the induction of apoptosis [26,27,36]. c-Fos/AP-1 has frequently been discussed to be involved in regulation of apoptotic signaling. Thus, *fas* ligand is AP-1 regulated [37]. Also, genes of the *fos/jun* family are inducible by many types of DNA damaging agents, which has been reported to play a role in apoptosis [38,39]. Cell death by apoptosis in c-*fos* knockout cells is a late response whereas Fos/AP-1 induction occurs immediately after genotoxic treatment. This deviation in time course might be explained by assuming that the immediate-early Fos/Jun/AP-1 induction exerts a protective effect by regulation of DNA repair and the progression of cells through S-phase. Indeed, c-*fos* knockout cells are impaired in the abolition of the DNA damage-induced DNA replication blockage [35]. This is related to an increased vulnerability to long-term genetic damage such as DNA breakage and chromosomal aberrations, which were found to be enhanced in c-Fos deficient cells [26,36]. Thus, the immediate-early response involving Fos/AP-1 might be critical in the regulation of DNA repair (either directly or *via* cell cycle checkpoint regulation), which could impact the apoptotic late response of cells.

12. Conclusions

DNA repair deficient mutants are generally hypersensitive to DNA damaging agents, responding with a high yield

[3] Dunkern, Roos and Kaina, unpublished data.

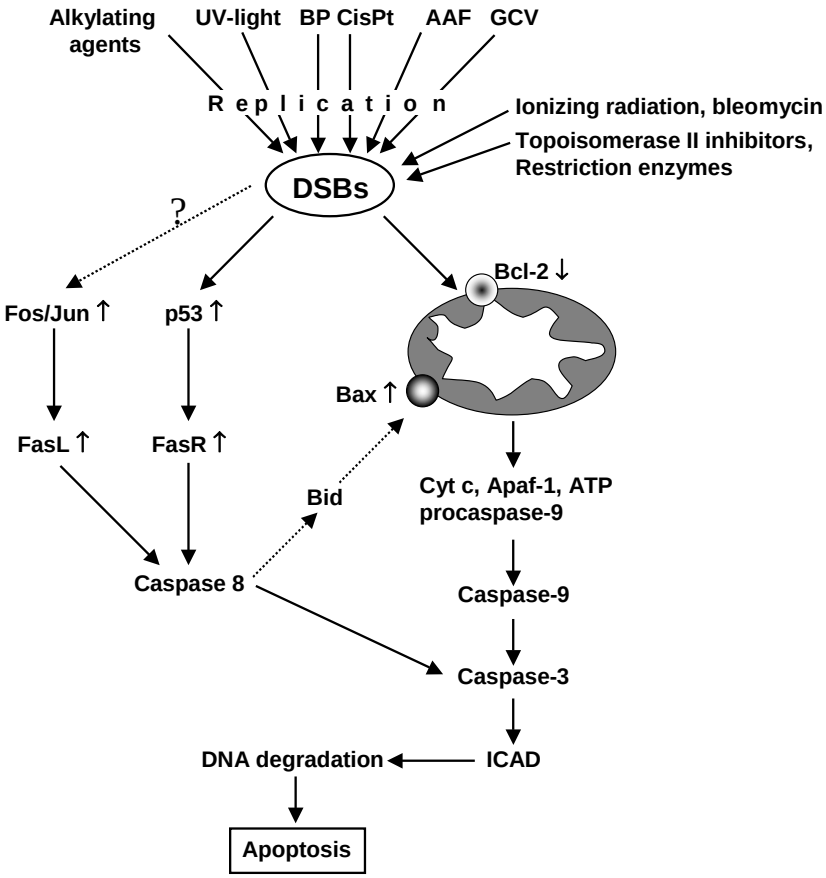

Fig. 3. Apoptotic pathways triggered by non-repaired DNA DSBs. Alkylating agents, oxidative stress, UV-C light, DNA incorporated GCV and agents inducing bulkyl lesions such as benzo(*a*)pyrene (BP), acetylaminofluorene (AAF) and cisplatin require DNA replication in order to form DSBs whereas IR, bleomycin, topoisomerase II inhibitors and electroporated restriction enzymes induce DSBs directly. DSBs are proposed to be the critical ultimate apoptosis inducing DNA lesion upon genotoxin treatment triggering either decline of Bcl-2 and activation of the mitochondrial damage pathway. They also provoke stabilization of p53 which stimulate the promoter of the *Fas* receptor gene, thus activating the Fas/CD95/Apo-1 apoptotic pathway with caspase-8 as the proximal activated caspase. DNA damage may also activate the MAPK pathway leading to upregulation of Fos and Jun (AP-1) which transcriptionally stimulate the *fas-L* gene. Whether DSBs directly activate Fos/Jun upregulation is unknown.

of apoptosis. DNA repair deficient experimental systems provided compelling evidence that particular DNA lesions such as O^6MeG, pyrimidine dimers and (6–4) photoproducts, incompletely repaired *N*-alkylation lesions and DNA crosslinks are able to trigger apoptosis. These primary DNA lesions, however, do not provoke this by themselves. Primary DNA lesions rather need to be converted *via* DNA replication into critical secondary lesions, which act as the ultimate apoptotic trigger. Therefore, DNA replication is an essential element in apoptosis provoked by many genotoxins (see Fig. 3). Based on available data, it is proposed here that the most critical ultimate apoptotic lesion arising from simple methylation and oxidative DNA lesions, UV-light and larger DNA adducts during DNA replication are DSBs. DSBs are highly efficient in inducing apoptosis which was verified by *Pvu*II electroporation provoking specifically DSBs and, as a late response, apoptosis. The apoptotic pathways presumably triggered by DSBs are outlined in Fig. 3. Whether chromosomal aberrations resulting from non-repaired or misrepaired DSBs also give rise to apoptosis (e.g. by loss or imbalance of survival factors) remains an open question, which will hopefully be answered in the future. Fibroblasts deficient in p53 or c-Fos are hypersensitive to various DNA damaging treatments including alkylating agents. Thus, these important DNA damage-inducible transcription factors exert a protective, anti-apoptotic affect in the fibroblast cell system, which is explained by up-regulation of DNA repair and checkpoint control allowing cells to recover from DNA damage.

Acknowledgments

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