How to Cope with DNA Damage Induced by Ionizing Radiation and Anti-Cancer Drugs?

Atsushi ENOMOTO and Kiyoshi MIYAGAWA*)

Laboratory of Molecular Radiology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

Ionizing radiation and chemotherapeutic agents induce many types of DNA lesions, of which DNA double-strand breaks (DSBs) are assumed to be the most deleterious. DNA damage response mechanisms encompass pathways of DNA damage signaling, DNA repair, cell cycle checkpoint arrest, and apoptosis. Increasing evidence suggests that these pathways function co-operatively to maintain genomic stability in the face of exogenous and endogenous DNA damage. The relative impact of one mechanism over another probably depends on the kinds of lesions, the cell cycle phase, and the cell or tissue type. The inability to respond properly to or to repair DSBs may lead to hypersensitivity to DNA damaging agents and genomic instability including chromosomal aberrations. Chromosomal instability, a state of continuous accumulation of chromosomal change, is a common feature of many human cancers and of chromosome instability syndromes with increased cancer susceptibility. Here, we review the DNA damage response and the links between deficiencies in response to DSBs and chromosomal instability.

§1. Introduction

Ionizing radiation and chemotherapeutic drugs produce a wide array of DNA lesions, including DNA base damage (base modification), single-strand breaks (SSBs), double-strand breaks (DSBs), sugar damage, DNA-DNA cross-links, and DNA-protein cross-links. For example, a cell exposed to 1 Gy of ionizing radiation will sustain 1000–2000 damaged bases, 800–1000 events of sugar damage, 1000 SSBs, approximately 40 DSBs, 30 DNA-DNA cross-links, and 150 DNA-protein links.[1] Of the many types of DNA damage in the cell, DSBs are the most dangerous because their repair is intrinsically more difficult than that of other types of DNA damage. DNA damage can also result from endogenously generated reactive oxygen species (ROS) and from mechanical stress on chromosomes, and DSBs can be generated when DNA replication forks encounter single-strand breaks or other types of lesions. Moreover, DSBs are produced to initiate recombination between homologous chromosomes during meiosis and occur as intermediates during developmentally regulated rearrangements such as V(D)J recombination.

Cells have evolved elaborate damage response mechanisms to cope with constant attacks on their DNA and to maintain genomic stability. These mechanisms involve quick transmission of a DNA damage signal to the checkpoint arrest, apoptotic or DNA-repair machinery. In mammalian cells, DNA damage signal transduction is mediated by the phosphatidylinositol-3-kinase-related protein kinase (PIKK) family, which phosphorylates substrates involved in regulation of checkpoint arrest, DNA

*) Corresponding author: miyag-tky@umin.ac.jp

repair or apoptosis.[2] Cell cycle arrest is necessary to give the cell sufficient time for repair, or to remove cells with excessive unrepaired DNA damage from the population by apoptosis, a form of controlled cell death.

Mammalian cells use two main mechanisms for DSB repair: non-homologous end joining (NHEJ) and homologous recombination (HR). HR is especially operative in the S/G2 phases of the cell cycle when a sister chromatid is available. NHEJ, which simply pieces together broken DNA ends, can function in all phases of the cell cycle. The inability to respond properly to or to repair DNA DSBs may lead to genomic instability, including chromosomal aberrations such as loss or gain of chromosomes and translocations, which in turn may lead to chromosomal instability and tumorigenesis. It is becoming evident that deficient responses to DSBs in signaling, checkpoint and DNA repair pathways are instrumental in the development of a number of cancers. There are also several human disorders with pronounced cancer predisposition that are characterized by defects in proteins that function in DNA-DSB responses. Here, we review the DNA damage response processes, including the DSB-induced cell cycle checkpoint, DSB repair, and apoptosis, and describe the contributions of components of each process in maintaining genomic stability and preventing cancer.

§2. Sensing DSBs and signal transduction

The DNA-damage response pathway consists of three components: damage sensors, signal transducers, and effectors (Fig. 1). The first step in the cellular response to DSBs is sensing of lesions, and this is followed by initiation of damage signaling. Two PIKKs, ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR), sense DNA damage or stalled replication forks in collaboration with other factors and initiate the signaling cascade.[2] Current evidence indicates that the MRE11, RAD50 and NBS1 (MRN) complex is one of the primary DSB sensors. The MRN complex, which has nuclease activity, binds at broken DNA ends and processes these ends prior to DNA repair. The MRN complex also functions in recruitment of ATM to DSB ends.[3] In the unstimulated state, ATM protein kinase is proposed to exist as a homodimer in which the kinase domain of one subunit faces the auto-phosphorylation site. In response to DNA damage, ATM is recruited to DSB sites by the MRN complex, and then a conformational change of the ATM protein occurs. This leads to autophosphorylation of ATM, causing dissociation of the homodimer to the active monomeric form.[2] Once activated, ATM phosphorylates a number of substrates, including checkpoint kinases CHK1 and CHK2, which in turn target other proteins to induce cell cycle arrest as effectors of ATM.[4] Cells derived from ataxia telangiectasia (AT), a human disorder conferred by mutations in ATM, have defects in inhibition of DNA replication after DNA damage that cause hypersensitivity to ionizing radiation.

ATR mainly responds to single-stranded regions of DNA (ssDNA) generated at stalled replication forks and other forms of DNA damage such as UV irradiation. ATR is constitutively associated with ATR-interacting protein (ATR-IP),[5] but for optimal activation of ATR several other proteins must be recruited to the ssDNA site. These include clamp-loading RAD17-containing complex, a PCNA-like sliding

clamp RAD9/HUS1/RAD1 (9-1-1 complex), and replication protein A (RPA), which binds to the ssDNA site and stabilizes the ssDNA. In response to DNA damage, the 9-1-1 complex is loaded on the DNA lesions by the RAD17-containing complex and then interacts with RPA, which in turn facilitates recruitment of the ATR/ATR-IP complex to DNA lesions that contain ssDNA through an interaction between the ATR/ATR-IP complex and RPA.[6] All these events are required for activation of ATR. Once activated, ATR phosphorylates multiple substrates overlapping with those of ATM, leading to induction of cell-cycle checkpoint arrest.[4),5)] As described above, there are distinct signaling pathways responsible for different types of DNA lesions.

Recent evidence indicates that modulators and adaptors promote protein-protein interactions that can facilitate ATM signaling. For instance, BRCA1, the product of the *breast cancer susceptibility gene 1*, has been linked to DNA-damage responses. BRCA1 has two tandem BRCT (BRCA1 carboxyl-terminal repeat) domains mediating protein-protein interactions, a feature that is shared with a number of other proteins involved in DNA-damage signaling. In fact, BRCA1 is part of the so-called BRCA1-associated genome surveillance complex (BASC) that includes putative DNA damage sensor machinery such as ATM and the MRN complex.[7] In this complex, BRCA1 may act as a scaffold protein that orchestrates the repair or checkpoint-signaling pathway, depending on the type of DNA lesion.

Another early step in the response to DSBs involves phosphorylation of the histone variant H2AX.[8] Phosphorylation of H2AX (termed gamma-H2AX) extends over mega base-pair regions of DNA from the break site and can be visualized as foci using antibodies specific for phosphorylated H2AX. There appears to be a close relationship between the numbers of DSBs and gamma-H2AX foci formed, and the rate of loss of gamma-H2AX foci correlates with the rate of DSB repair. Phosphorylated H2AX is thought to function as an important factor that facilitates retention of proteins such as the MRN complex and BRCA proteins at DSB sites. Taken together, the steps of PIKK signaling include sensor proteins such as the MRN complex, adaptor proteins that facilitate PIKK signaling, and effector kinases such as CHK1 and CHK2 that transduce the signal to downstream proteins.

§3. DNA-damage-induced checkpoint regulation of the cell cycle

Checkpoint arrest after DNA damage has two potential impacts: it allows additional time for DNA repair before cell cycle progression and it can permanently prevent proliferation of severely damaged cells (Figure 1). In response to DSBs, checkpoint machineries are activated, resulting in cell-cycle arrest at DNA-damage response checkpoints. These checkpoints have been identified at the G1/S, intra-S, and G2/M boundaries, and initiation of the activities of PIKKs, ATM and ATR is currently thought to be the first step in activation of the checkpoint machinery.[9]

ATM and ATR regulate the G1/S checkpoint after DNA damage, at least in part by regulating activation and stabilization of p53. ATM and ATR phosphorylate p53 directly or indirectly through CHK1 and CHK2, and phosphorylation of p53 leads to its stabilization by interfering with p53 binding to MDM2 (mouse double minute

2), a negative regulator of p53 that targets p53 for degradation through proteasome machinery. The p53 protein acts primarily as a transcriptional factor that is activated by DNA damage. The key transcriptional target of p53 is p21CIP1/WAF1, an inhibitor of cyclin-dependent kinases, which silences the G1/S promoting kinase Cyclin E/CDK2 and thereby causes G1 arrest.[10] The ATR/CHK1 pathway also causes degradation of CDC25A, an activator of Cyclin E/CDK2 kinase, through a ubiquitin-dependent proteasome-mediated pathway and consequently leads to inhibition of the Cyclin E/CDK2 kinase complex. Thus, at the G1/S checkpoint, ATM (ATR) and CHK2 (CHK1) play a critical role in regulating the p53/MDM2/p21-dependent pathway and the CDC25A degradation cascade.

The intra-S-phase checkpoint networks are also in part controlled by the ATM/ATR signaling machinery. CHK1 and CHK2 activated by ATM/ATR phosphorylate CDC25A, and subsequent degradation of CDC25A leads to inactivation of CDK2, as described above. Then, inhibition of CDK2 activity blocks loading of CDC45 onto chromatin, which is required for recruitment of DNA polymerase α into assembled pre-replication complexes.[11] Thus, inhibition of CDK2 activity prevents the initiation of new origin firing.

The G2 checkpoint prevents initiation of mitosis when cells experience DNA damage during G2 or when they progress into G2 with unrepaired damage that was inflicted during the preceding S or G1 phases. The critical target of the G2 checkpoint is CyclinB/CDK1 kinase, which promotes the initiation of mitosis. In response to DNA damage, activation of CyclinB/CDK1 kinase is inhibited by the ATM (ATR)/CHK2 (CHK1) signaling pathway through degradation and down-regulation of the CDC25 family of phosphatases that normally activate CDK1 at the G2/M boundary.[9),12] Thus, ATM and ATR are involved in all checkpoints of the cell cycle.

§4. Repair of DNA double strand breaks

Once cells have sensed DSBs, the DNA repair machinery is activated and recruited to the breaks (Fig. 1). There are two major pathways for the repair of DSBs: NHEJ and HR. NHEJ is a simpler and more error-prone mechanism. In essence, NHEJ rejoins the two severed DNA ends in a sequence independent fashion.[13] This pathway is most precise for simple breaks such as blunts ends, but not all DNA ends are readily ligated. Ends that are not compatible require processing before proper rejoining can proceed. The proteins that are required for NHEJ include the heterodimer Ku70/Ku80 and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). Heterodimers of Ku form a ring structure that binds specifically to the ends of DNA DSBs, and then this DNA-Ku complex recruits DNA-PKcs to the site, generating the DNA-PK holoenzyme complex and activating its kinase activity.[14] The activated DNA-PK complex recruits Artemis, which has an endonuclease activity necessary for DNA-end processing.[15] Ku also recruits the XRCC4/DNA ligase IV complex, which ligates the processed DNA ends.[16] Thus, the DNA-PKcs/Ku complex has a primary DSB recognition role and then facilitates recruitment and activation of other NHEJ components such as Artemis and XRCC4/DNA ligase IV. The majority of DSBs induced by ionizing radiation are repaired by NHEJ in an

ATM- and ATR-independent manner. Conversely, ATM-dependent signaling and cell cycle-checkpoint arrest occur efficiently in NHEJ-deficient cells. Current evidence indicates that core NHEJ occurs independently of ATM signaling, although a subset of DSBs requires ATM for Artemis-dependent end-processing in NHEJ.[17),18)]

NHEJ also rejoins DSBs that are introduced during V(D)J recombination. During early B and T cell development, exons that encode immunoglobulin and T cell receptor variable regions are assembled from variable (V), diversity (D), and joining (J) segments via V(D)J recombination, in which a high mutation rate results in the evolutionary advantage of immunological diversity capable of responding to a wide array of antigens.[19)] The phenotype of mice with DNA-PKcs deficiency is that of severe combined immunodeficiency (SCID) syndrome due to impaired V(D)J recombination. Inherited defects in NHEJ account for about 15% of human SCID, and nearly all of these are null mutations of Artemis. Moreover, mutations in any of the key genes regulating NHEJ result in significant hypersensitivity to ionizing radiation.[20)] Therapeutically, inhibitors of NHEJ could be great value as radiation sensitizers or in combination chemotherapy strategies.

The second DNA DSB repair system, HR, uses an undamaged sister homolog as a template, thereby providing a process capable of achieving high fidelity even if sequence information is lost at the break site. This error-free process can be accomplished by finding a homologous sequence, generally encompassing 100bp or more and preferably in a sister chromatid. Therefore, in mammalian cells HR is favored in S and G2 phases because an intact sister chromatid is readily available to serve as a template. Interestingly, the S phase corresponds to the most radio-resistant phase of the cell cycle, suggesting that DSBs are accurately and rapidly repaired by an intact HR system. The HR pathway is initiated by resection of DNA ends to generate a single-stranded region of DNA, followed by invasion of the template strand, which creates crossed DNA strands or "Holliday junctions".[21)] Holliday junctions further stabilize the joined molecules, and high fidelity DNA synthesis occurs using the sister strand as a template, followed by branch migration and finally resolution of the Holliday junction.[22)] RAD51, a central player in HR, is loaded onto single-stranded DNA. RAD51 catalyzes the search for homologous sequences and promotes strand invasion. The interaction between RAD51 and BRCA2 is critical for biological functions of both molecules.[23)] Other proteins involved in HR include XRCC2, XRCC3, RAD51B, RAD51C, and RAD51D. Most of these proteins have been recognized as recombination mediators based on their ability in biochemical assays to assist RAD51 function. Recently, RAD54 has been shown to facilitate Holliday junction migration and allow for separation of the DNA strands after completion of synthesis.[24)]

HR also has a key role in repairing DSBs that arise as a result of replication-fork stalling. In general, cells with defective HR, but intact NHEJ, are moderately radiosensitive, but demonstrate remarkable hypersensitivity to DNA-crosslinking agents.[25)] These results suggest that the main function of HR is to repair DSBs at replication forks, whereas NHEJ repairs DSBs that have been generated elsewhere in the DNA.

§5. Genomic instability and cancer

Cells respond to DNA damage by activating a complex DNA-damage-response pathway that induces cell-cycle arrest, activation of DNA repair machinery, and, under some circumstances, apoptosis. However, an inability to respond properly to or to repair DNA damage can lead to genetic instability, which in turn increases the risk of cancer (Fig. 1). Two main forms of genomic instability are associated with tumors. The mutational instability phenotype is characterized by small sequence changes, including base substitutions and insertions/deletions of a few nucleotides. In contrast, the chromosomal instability phenotype is characterized by gross rearrangement of chromosomes, the loss or gain of whole chromosomes or chromosomal fragments, and amplification of fragments. Chromosomal aberrations are typically subdivided into chromatid breaks and gaps, chromosome breaks and gaps, triradials, quadriradials, and dicentric chromosomes. The loss of large regions of a chromosome may lead to inactivation of tumor suppressor genes, whereas amplification of chromosome regions can result in activation of proto-oncogenes or induction of multi-drug resistance after cytostatic drug treatment.[26] These chromosomal aberrations can arise from defective repair of DSBs, from entry of cells into mitosis before DSBs are repaired, or from rejoining of DSBs on two different chromosomes. The following evidence for links among DSB repair systems, genomic instability and cancer predisposition syndromes has emerged: chromosomal instability induced by X-irradiation is exacerbated in DNA repair-deficient cells, many chromosomal translocations in lymphoid tumors include breakpoints in the immunoglobulin or T-cell receptor loci, and deficiencies in DSB-signaling proteins in patients and mouse models result in gross chromosomal instability.[27]

One of the most important disease examples is ataxia telangiectasia (AT), which is caused by a deficiency of ATM kinase. This disorder is characterized by hypersensitivity to ionizing radiation, progressive neurodegeneration, immunodeficiency, a high incidence of chromosomal translocations, and frequent lymphoreticular malignancy (lymphomas and leukemias). AT carriers (ATM heterozygotes) constitute approximately 1% of the general population and are believed to have a 3- to 5-fold increased risk of developing breast cancer.[28] The increased cancer susceptibility in AT patients is at least partly due to chromosomal instability. Furthermore, homozygous *ATM* knockout mice show a high incidence of lymphoid tumors and chromosomal instability with gaps, breaks and translocations at T-cell receptor loci, indicating that DSBs generated by the V(D)J recombination machinery are at least partly responsible for these chromosomal aberrations.[29] Nijmegen breakage syndrome (NBS) is an AT-liked disorder that is caused by a deficiency in NBS1 protein in humans[30] and characterized by developmental defects, hyperradiosensitivity, and predisposition to cancer. *MRE11* mutations have been found in patients with another AT-like disorder, AT-LD,[31] and the overlap between the phenotypes of AT, NBS, and AT-LD indicates that ATM and the MRN complex function in the same signal transduction pathway.

Since HR is considered to be more accurate than NHEJ in DNA repair, unregulated HR can play a role in carcinogenesis. Bloom syndrome (BS) is a reces-

sive genetic disorder associated with genomic instability, and is characterized by growth retardation, immunodeficiency, and an increased risk of developing cancer. BS is caused by mutations in BLM helicases, which are members of a class of DNA-unwinding enzymes that participate in the migration of Holliday junctions in HR. Cells from Bloom syndrome patients show abnormally high levels of sister chromatid exchanges (SCE) through the HR pathway.[32] Thus, the genomic instability and cancer predisposition phenotype in Bloom syndrome is at least partly due to an increase in the frequency of somatic recombination. The inheritance of germline mutations in *BRCA1* or *BRCA2* confers susceptibility to breast and ovarian cancers with a lifetime risk of up to 60%.[33] In most breast cancers arising in BRCA1/2 mutation carriers, inactivation of the wild-type allele has occurred by loss of heterozygosity, thus abolishing normal protein expression. The loss of BRCA functions causes a significant increase of genomic alterations, including chromosomal aberrations, which may be caused by a deficiency in the repair of DNA DSBs.[34] BRCA1- and BRCA2-deficient cells are hypersensitive to agents that cross-link DNA, such as mitomycin C. An increase of genomic instability is particularly obvious in combination with a mutation in the tumor suppressor gene p53, because of abrogation of cell-cycle arrest or a reduced rate of apoptosis. In fact, familial-BRCA1 and -BRCA2 tumors carry a higher frequency of *p53* mutations than control sporadic cancers.[35] The importance of the role of p53 in maintaining genome stability is exemplified by findings that this molecule is mutated in approximately 50% of tumors. Li-Fraumeni syndrome is an inherited human cancer predisposition condition that is generally caused by heterozygous mutations in p53.

Deficiencies in NHEJ also lead to an increased risk of cancer with enhanced chromosomal instability. Artemis is mutated in a subset of human SCID (severe combined immunodeficiency) patients, with the condition referred to as RS-SCID.[36] Such patients with hypomorphic mutations in the *Artemis* gene display hypersensitivity to ionizing radiation and have been found to develop thymic lymphomas.[37] Cells from Artemis knockout mice exhibit significantly higher levels of spontaneous chromosomal aberrations such as chromosomal fusions and detached centromeres. Moreover, cells from patients with inherited hypomorphic mutations in *DNA ligase IV* are hyperradiosensitive, impaired in DSB repair, and display significantly elevated chromosomal breaks after ionizing radiation.[38] The deficiency of DNA ligase IV functions has been linked to predisposition to multiple myeloma and leukemia.[39]

Since checkpoints serve to prevent cells with an excessive level of damage from entering mitosis, loss of a checkpoint can enhance chromosomal aberrations and the risk of cancer. *CHK1* haploinsufficiency results in three distinct phenotypes that can contribute to tumorigenesis: inappropriate S-phase entry, accumulation of DNA damage during replication, and failure to restrain mitotic entry.[40] Homozygous inactivation of *CHK2* does not cause spontaneous development of tumors; however, a lack of CHK2 enhances skin tumorigenesis induced by carcinogen exposure. Inherited mutations in one allele of *CHK2* have been found in some families with extremely cancer-prone Li-Fraumeni-like syndromes that do not carry mutations in p53.[41]

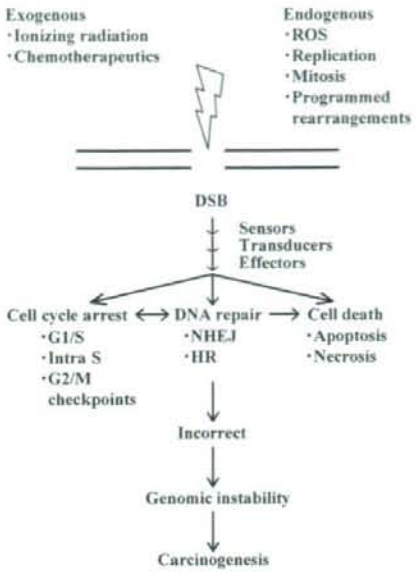

Fig. 1. General organization of the DNA-damage response pathway. DNA double-strand breaks (DSBs) result from exogenous stimuli such as ionizing radiation and also from various cellular processes including DNA replication and V(D)J recombination. Cells have evolved protective responses to cope with the constant attack on their DNA and to maintain genome stability. The presence of DSBs is recognized by a sensor, which transmits the signal to a series of downstream effector molecules through a transduction cascade to activate signaling mechanisms for cell cycle arrest, DNA repair or cell death. Extensive DNA damage may result in cell death, which is subdivided into two modes based on morphological criteria: necrosis or apoptosis. Necrosis is a passive form of cell death, and apoptosis is a form of highly regulated cell death. An alternative protective mechanism is provided by the combination of cell-cycle checkpoints and DNA-repair. Checkpoint arrest after DNA damage allows additional time for DNA repair to occur before cell cycle progression and this can prevent proliferation of severely damaged cells. DNA repair then restores the integrity of the genome during cell cycle arrest. There are two major pathways for repair of DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ rejoins the two DNA ends in a sequence-independent fashion, but is the more error-prone mechanism. HR uses an undamaged sister homolog as a template, thereby providing an error-free process. The fidelity of repair is of great importance to the fate of the cell. Inaccurate repair leads to genomic instability via mutations and chromosomal aberrations, which in turn contribute to carcinogenesis.

§6. DNA-damage-induced cell death

DNA-damage-induced cell death is classified into two types: interphase cell death and reproductive cell death.[42] Interphase cell death is defined as 'cell death before reaching the first mitosis' and is often observed in thymocytes and lymphoid cells

after treatment with DNA-damaging agents. In contrast, reproductive cell death occurs after one or more divisions, and this process seems to be related to DNA-damage-induced chromosomal aberrations. Reproductive cell death often occurs following high doses of irradiation. DNA-damage-induced cell death is also classified into two modes by morphological criteria: apoptosis and necrosis.[43] Apoptosis is a form of controlled cell death that has come to be used synonymously with the phrase 'programmed cell death'. Apoptosis is an important process in tissue homeostasis, and cells also undergo apoptosis in response to a variety of stimuli, including DNA damage, cytotoxic agents, and ligand binding.[44)–47] The morphological features of apoptosis include nuclear condensation and fragmentation, cleavage of chromosomal DNA into internucleosomal fragments, loss of mitochondrial membrane potential, membrane blebbing, and cell shrinkage and disassembly into membrane-enclosed vesicles referred to as apoptotic bodies.[48] Apoptotic bodies are recognized and removed by phagocytic cells, thus resulting in deletion of dying cells. Moreover, the execution of apoptosis requires energy in the form of adenosine triphosphate (ATP). In contrast, necrosis is thought of as a passive form of cell death and is usually considered to be unregulated. Necrosis is the end result of a bioenergetic catastrophe with ATP depletion and is initiated mainly by toxic insults or physical damage.[49] A classical positive definition of necrosis based on morphological criteria includes early plasma membrane rupture and dilatation of cytoplasmic organelles, in particular mitochondria, resulting in release of cellular contents and proinflammatory molecules that induce inflammation. In necrosis, changes in nuclear morphology such as chromatin condensation and nuclear DNA fragmentation are not observed. The type of cell death that occurs depends on cell types, kinds of stimuli, oncogene expression, and the extent of damage.

§7. Mitochondrial function in cell death

Mitochondria are often referred to as the powerhouse of the cell, because their function is to supply ATP produced by the respiratory chain in the mitochondrial inner membrane as a source of energy. However, in the last 10 years it has been become increasingly clear that mitochondria play a central role in processes that lead to cell death.[50] Exposure of cells to toxic agents induces various forms of mitochondrial damage and dysfunction, such as mitochondrial membrane permeabilization (MMP), loss of mitochondrial membrane potential, and release of apoptosis-promoting factors including cytochrome *c*.[51] DNA DSBs induces the translocation of histone H1.2 from the nucleus into the cytosol, where histone H1.2 induces the release of cytochrome *c* from mitochondria.[52] Cytochrome *c* normally functions as part of the respiratory chain to maintain the mitochondrial transmembrane potential, but when released into the cytosol it becomes a critical component of the apoptosis execution machinery. MMP leads to disruption of mitochondrial structure and function: outer MMP leads to leakage of intermembrane proteins from mitochondria and inner MMP is linked to bioenergetic failure caused by loss of the mitochondrial transmembrane potential. Such damage is generally considered to be the point of no return in programmed cell death.

§8. Execution of apoptosis by caspases

Cells that harbor unrepaired DNA damage are removed from the population by apoptosis. Apoptotic cell death is not merely a result of inactivation of the genome, but rather is based on a series of complex enzymatic reactions in proteolytic cascades. Genetic studies of developmental programmed cell death in *C. elegans* have demonstrated that Ced-3 cysteine protease is required for execution of cell death.[53] This then led to identification of its mammalian homolog, interleukin-1β-converting enzyme-like protease (ICE). Currently, at least 14 mammalian homologs of *ced-3* have been identified. These proteases are now termed caspases (cysteinyl aspartate-specific proteinase),[54] and each contains a conserved QACXG pentapeptide surrounding the active site cysteine.[55] In addition to their sequence similarity to Ced-3, the members of the caspase family of proteases have several unifying characteristics. Caspases are constitutively expressed as an inactive proenzyme composed of a variable-length amino-terminal prodomain, a large subunit, and a small subunit. Activation of caspases requires proteolytic processing of the proenzyme at specific aspartate residues, thereby resulting in removal of the prodomain and formation of a heterodimer containing one large and one small subunit.[55] The active caspase is a tetramer composed of two such heterodimers. Caspases are classified into two types: initiator and effector caspases. Enzymatic activation of initiator caspases such as Caspase-8 and -9 leads to proteolytic activation of downstream effector caspases such as Caspase-3, -6 and -7. In particular, irradiation or DNA-damaging agents preferably activate Caspase-9 as an initiator caspase, and a deficiency of Caspase-9 results in resistance to apoptosis induced by radiation or DNA damage.[56] The release of cytochrome *c* from mitochondria into the cytosol acts as a trigger for formation of the Caspase-9/Apoptotic protease activating factor-1 (Apaf-1)/cytochrome *c* complex (referred to as the apoptosome) in the presence of dATP or ATP, leading to initiation of the Caspase-9 cascade. Apaf-1 is the mammalian homolog of *C. elegans* Ced-4, and Ced-3-induced cell death requires the function of Ced-4.[57] In contrast, Caspase-8 is an initiator caspase of death receptor-mediated apoptosis. Caspase-8 contains an N-terminus with a FADD (Fas-associating protein with Death Domain)-like death effector domain and provides a direct link between cell death receptors and caspases. Activated caspases precipitate an irreversible commitment to apoptotic cell death by cleaving a number of substrates, including activating caspases themselves through cleavage at an aspartate at the carboxy terminus (the P1 site). Caspases cleave a variety of important cellular proteins, such as apoptosis regulators and DNA repair proteins, leading to loss of function. At present, almost 300 proteins have been identified as substrates of caspases.[58),59] For example, cleavage of the DNA-dependent protein kinase catalytic subunit (DNA-PK) and poly(ADP-ribose) polymerase (PARP) results in a loss of catalytic activity, and caspase-mediated cleavage of BCL-2 and BCL-x_L abrogates their anti-apoptotic activities. Moreover, caspases are required for some morphological changes of cells undergoing apoptosis. For example, cleavage of the nuclear envelope protein Lamin results in nuclear lamina disassembly. In host defense, caspases serve to transmit

and amplify death signals through a protelytic cascade.

§9. BCL-2 related proteins: regulators of apoptosis

BCL-2 proteins are evolutionarily conserved regulators of apoptosis.[60] More than 30 members of the BCL-2 family have been identified. Proteins of this family include both anti-apoptotic and pro-apoptotic members and directly regulate the release of mitochondrial apoptotic factors. The best-characterized anti-apopototic proteins, BCL-2 and BCL-X_L, which are composed of four BH (BCL-2-homology) domains, prevent interaction and activation of proapoptotic members of the family, and thereby inhibit release of cytochrome *c* and subsequent caspase activation. Pro-apoptotic members are divided into multidomain pro-apoptotic BCL-2 proteins such as BAX and BAK, which lack the BH4 domain necessary for an inhibitory effect on apoptosis, and BH3-only proteins such as BID, BAD, PUMA, NOXA, BIM and BMF, which share homology with the BCL-2 family only in the BH-3 domain.[61] These multidomain- and BH3-only proteins function co-operatively to promote apoptosis. In unstimulated cells, BAX exists as a monomer either freely in the cytosol or loosely attached to the outer mitochondrial membrane. Activator BH-3-only proteins (BIM and BID) are widely thought to stimulate a conformational change of BAX into an active form, its translocation to mitochondria, and the BAX/BAK interaction, thereby promoting mitochondrial membrane permeabilization (MMP). Although a deficiency in either BAX or BAK does not affect apoptosis, a deficiency in both results in resistance to apoptosis induced by radiation or DNA-damaging agents.[62] Moreover, mice that are genetically deficient in either BH-3-only protein PUMA or NOXA show resistance to apoptosis.[63),64] These results indicate that BH-3-only proteins promote BAX/BAK interaction, leading to membrane permeabilization of mitochondria and the release of cytochrome *c*, thereby inducing apoptosis.

§10. Signal transduction in DNA-damage-induced apoptosis

Unless DSBs are correctly repaired, they trigger apoptosis. However, it is unclear how signaling is transmitted downstream to executors of apoptosis. Recently, considerable information has been accumulated on signal transduction mechanisms in DNA-damaged-induced apoptosis. One of the most important signal transducers is the DSB sensor molecule, ATM. Once activated, ATM phosphorylates various downstream substrates such as checkpoint kinases CHK1/CHK2 and p53, a central player in DNA-damaged-induced apoptosis in mammalian cells. The p53 protein is a tumor suppressor gene product that acts primarily as a transcription factor. In response to DNA damage, p53 induces transcriptional activation of pro-apoptotic factors such as BAX, PUMA, NOXA, BID, APAF-1 and FAS,[65] and silencing of each of these genes induces partial resistance to p53-dependent apoptosis.

The amount of p53 protein present in unstressed cells is low and this is determined by its rate of degradation, rather than by translation from mRNA. While the exact mechanism of stabilization of p53 remains unclear, it involves a series of post-translational modifications of both p53 and the ubiquitin ligase MDM2. Degradation

of p53 is ensured by auto-regulatory negative feedback loops in the form of ubiquitin ligases, which ubiquitinate substrates prior to proteasome degradation.[66] MDM2 regulates the activity of p53 by ubiquitination, leading to transport of p53 to the cytoplasm and proteasomal degradation. DNA damage induces post-translational modifications of p53 and MDM2, including phosphorylation, which facilitates dissociation of the MDM2-p53 complex, and it has been suggested that phosphorylation of p53 protects it from degradation and enhances its stability and affinity for sequence-specific DNA binding sites. The most frequently described phosphorylation is at Ser 15, and this reaction is in part mediated by ATM and occurs rapidly in response to DSBs.[67] Indeed, ATM mediates phosphorylation at multiple sites on p53 in response to ionizing radiation. On the other hand, MDM2 also self-ubiquitinates, and exposure of cells to DNA-damaging agents causes an increase in self-ubiquitination and degradation, thus favoring p53 stabilization. It is believed that at low levels of DSBs only a minor function of p53 that is sufficient to induce transcription of the *p21* gene is activated, causing cell cycle arrest. With higher levels of DSBs, p53 accumulates above a particular threshold and can activate transcription of pro-apoptotic genes such as *PUMA* or *NOXA*.[61] The cellular response to p53 depends on cell type, the expression level of p53, and the extent of phosphorylation of p53.

Irradiation also causes various toxic events such as ROS production, lipid peroxidation and lipid second messenger ceramide generation. These events often induce apoptosis via a p53-independent pathway. Proteins in the mitogen-activated protein kinase (MAPK) signal transduction pathway, which is a conserved cascade of protein kinases, are critical mediators of the response of cells to a variety of extracellular changes and are involved in survival and cell death.[68),69] To date, various MAPK family molecules have been identified, and each is activated by a distinct signal and appears to be coupled to different biological responses. The c-Jun N-terminal kinase (JNK), a member of the MAPK family, is strongly activated by a wide variety of stimuli including irradiation, chemotherapeutic agents, ROS, and ceramide.[70] In mammals, there are 3 *JNK* genes: *JNK1, JNK2* and *JNK3*. In response to apoptotic stimuli, JNKs phosphorylate and activate transcription factors such as c-Jun and ATF-2, which induce transcription of target genes that include pro-apoptotic genes. JNK deficiency results in resistance to apoptosis induced by radiation- or DNA-damaging agents, indicating that JNKs play an important role in DNA-damage-induced apoptosis.[71] Also, a recent report demonstrated that activated JNKs are translocated to mitochondria and directly phosphorylate pro-apoptotic BCL-2 family proteins, leading to induction of apoptosis.[72] These results indicate that JNKs regulate apoptosis in both a transcriptional- and a non-transcriptional-dependent manner.

§11. Apoptosis and cancer

Apoptosis is a fundamental cellular homeostasis mechanism that ensures correct development and roles of multi-cellular organisms. The failure of correct execution of apoptosis causes various diseases, including cancer. During cancer development, modifications and imbalances may arise in the apoptotic machinery. For instance,

human papilloma viruses (HPV), which have been implicated in cervical cancer, produce an oncoprotein (E6) that binds and inactivates p53. Epstein-Barr virus (EBV), which is associated with some lymphomas, produces two proteins (BALF1 and BHRF1) that are similar to the anti-apoptotic protein BCL-2,[73] and some melanoma cells avoid apoptosis by down-regulating expression of apoptotic protease activating factor-1, APAF-1.[74] These imbalances in the apoptotic machinery prevent cells from executing apoptosis, which ultimately leads to inappropriate cell proliferation and malignant progression. Moreover, chromosomal instability or tumor incidence is enhanced in combination with a mutation in the tumor suppressor gene *p53* because of abrogation of cell cycle arrest or a reduced apoptosis rate. Indeed, Artemis knockout mice display only a minor elevated incidence of cancer, but this is increased dramatically in a p53 knockout background.[75] Bi-allelic disruption of *BRCA1* in mouse results in embryonic lethality, whereas increased mammary and lymphoma carcinogenesis has been observed in combination with p53 disruption.[76] These results indicate that DNA repair defects confer genomic instability, which is synergistic with checkpoint and/or apoptotic defects.

Concluding remarks

Advances in genetics coupled with increased understanding of DNA damage responses have led to identification of several genetic disorders that are conferred by mutations in genes that function in damage-response pathways. These disorders and animal models have identified the physiological roles of genes and the links with other pathways, and provided insights into the association between defects in DNA damage responses and cancer. Further elucidation of DNA damage response mechanisms will have important implications in cancer management and treatment.

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Int. J. Cancer: **00**, 000–000 (2009)

SHORT REPORT

Functional evidence for Eme1 as a marker of cisplatin resistance

Yoshitaka Tomoda[1,2], Mari Katsura[1], Miyuki Okajima[1], Noriko Hosoya[1], Nobuoki Kohno[2] and Kiyoshi Miyagawa[1]*

[1]*Laboratory of Molecular Radiology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*

[2]*Department of Molecular and Internal Medicine, Division of Clinical Medical Science, Graduate School of Biochemical Science, Hiroshima University, Hiroshima, Japan*

The ability to predict cisplatin sensitivity in tumors has been expected to greatly improve the outcome of cancer therapy, because the drug is frequently used in a variety of tumors. Although ERCC1 and other repair proteins have been investigated as markers of cisplatin resistance, reliable markers are still needed. Here, we demonstrate that Eme1 levels can predict cisplatin sensitivity more accurately than ERCC1 or Rad51 levels in a variety of human cancer cell lines. Eme1 forms a heterodimeric protein complex with Mus81 and functions as a structure-specific endonuclease. Haploinsufficiency of *Eme1* led to hypersensitivity to cisplatin in the colon cancer cell line HCT116. On the basis of this finding, we examined the relationships between levels of proteins involved in the repair of interstrand cross-links and cisplatin sensitivity in human tumor cell lines with a variety of origins. Although ERCC1, Rad51 and Mus81 levels correlated with sensitivity to some extent, the clearest correlation was observed with Eme1. Tumors with low Eme1 levels were more sensitive to the drug than tumors with high levels. This suggests that the measurement of Eme1 in tumors may be more informative for cisplatin-based chemotherapy than that of the currently available markers.

Key words: Eme1; Mus81; ERCC1; cisplatin sensitivity; DNA repair

Cisplatin and its analogues are chemotherapeutic drugs used widely in cancer treatment.[1] However, although these drugs benefit some patients, other patients suffer from their toxicity, such as nephrotoxicity, without experiencing their benefits. To resolve such clinical problems, the identification of markers that can predict who might benefit and who might not has been awaited since the introduction of cytotoxic chemotherapy for the treatment of cancer.

Cisplatin forms interstrand cross-links (ICL), which stall the progression of replication forks during DNA replication.[2,3] The DNA adducts formed by cisplatin are converted to DNA double-strand breaks (DSB). Subsequently, a single-strand DNA on the broken strand across the ICL is incised, resulting in the release of the lesion from the strand. The gapped DNA is assumed to be filled in by translesional synthesis. After the ICL is excised, the lesion is repaired by homologous recombination.

The XPF-ERCC1 complex is involved in the repair of ICL by incising DNA near the lesion.[4] Cells deficient in *ERCC1* are, therefore, hypersensitive to ICL-inducing agents.[5] On the basis of this finding, the relationship between levels of ERCC1 and sensitivity to cisplatin in tumors has been extensively examined.[6] These studies led to a large-scale clinical study demonstrating that non-small-cell lung cancer (NSCLC) patients with low ERCC1 levels benefit from cisplatin-based adjuvant chemotherapy.[7] Thus, ERCC1 is regarded as a marker of cisplatin resistance in tumors. However, ERCC1 levels alone cannot accurately predict sensitivity.

Rad51 plays a central role in homologous recombination at early stages. Because cells deficient in homologous recombination are hypersensitive to cisplatin, Rad51, like ERCC1, has been investigated as a marker of cisplatin resistance.[8] Recent evidence suggests that Rad51 is a potential marker of cisplatin resistance in NSCLC.[9] Despite these findings, it is still difficult to predict cisplatin sensitivity satisfactorily.

The Mus81-Eme1 complex was shown to induce DSB near ICL *via* structure-specific endonuclease activity, suggesting that the complex plays a role in the repair of ICL.[10] Consistent with this finding, murine normal cells deficient in *Mus81* or *Eme1* were shown to be hypersensitive to mitomycin C (MMC) and cisplatin.[11–13] Furthermore, the haploinsufficiency of *Mus81* led to hypersensitivity to MMC and cisplatin but not to other DNA-damaging agents in the human colon cancer cell line HCT116.[14] Additionally, the haploinsufficiency of *Eme1* led to hypersensitivity to MMC in the same cells.[14] These findings suggest that Mus81 and Eme1 may be the potential markers of cisplatin resistance in human tumors.

We show here that Eme1 is an appropriate marker of cisplatin resistance in human tumors. First, we confirmed that the haploinsufficiency of *Eme1* led to hypersensitivity to cisplatin in HCT116 cells. Second, Eme1 protein levels were examined in a variety of human tumor cell lines. They were well correlated with Mus81 levels but not with ERCC1 or Rad51 levels. Third, cell survival assays revealed that Eme1 protein levels predicted cisplatin sensitivity most accurately. These observations indicate the promise of Eme1 measurement in human tumors for individualized cancer therapy.

Material and methods

Cell culture

AN3CA, Du145, HeLa, HepG2, LS180, MCF7, T47D and A549 cells were cultured in DMEM with 10% fetal calf serum (FCS). HCT116 and SkBr3 cells were cultured in McCoy's 5A medium with 10% FCS. DLD1, Jurkat, K562, U937, KCL22, H358 and H522 cells were cultured in RPMI with 10% FCS. HT1080 cells were cultured in MEM with 10% FCS. These tumor cells were obtained from the American Type Culture Collection. Telomerase-immortalized retinal pigmented epithelial (RPE) cells were cultured in DMEM/F12 with 10% FCS, 2 mM L-glutamine and 0.348% sodium bicarbonate. Telomerase-immortalized human mammary epithelial (HME) cells were cultured in MCDB 170 medium with 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 10 ng/ml hEGF and 5 μg/ml insulin. RPE and HME cells were purchased from Clontech.

Western blot analysis

Whole cell extracts were prepared in lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA pH 8.0, 0.5% Nonidet P-40, 1 mM PMSF, 50 nM cantharidin, 5 nM microcystin and 2 μg/ml aprotinin). After SDS-PAGE, the proteins were transferred

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant number: 18012012; Grant sponsor: Ministry of Health, Labor and Welfare of Japan; Grant number: 19140401.

***Correspondence to:** Laboratory of Molecular Radiology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Fax: +81-3-5841-3013. E-mail: miyag-tky@umin.ac.jp

Received 20 August 2008; Accepted after revision 9 December 2008
DOI 10.1002/ijc.24268
Published online 00 Month 2009 in Wiley InterScience (www.interscience.wiley.com).

to PVDF filters. Non-specific binding sites were then blocked by immersing the filters in 5% non-fat dried milk, 0.1% Tween 20 in Tris-buffered saline for 60 min. The primary antibodies were anti-Eme1 (MTA31 7h2/1, Santa Cruz Biotechnology), anti-Mus81 (ab14387, Abcam), anti-ERCC1 (FL-297, Santa Cruz Biotechnology), anti-Rad51 (Ab-1, Calbiochem) and anti-Cdk2 (M2, Santa Cruz Biotechnology). Horseradish peroxidase-labeled antibodies were used as secondary antibodies (GE Healthcare). Blots were detected using ECL reagents (GE Healthcare).

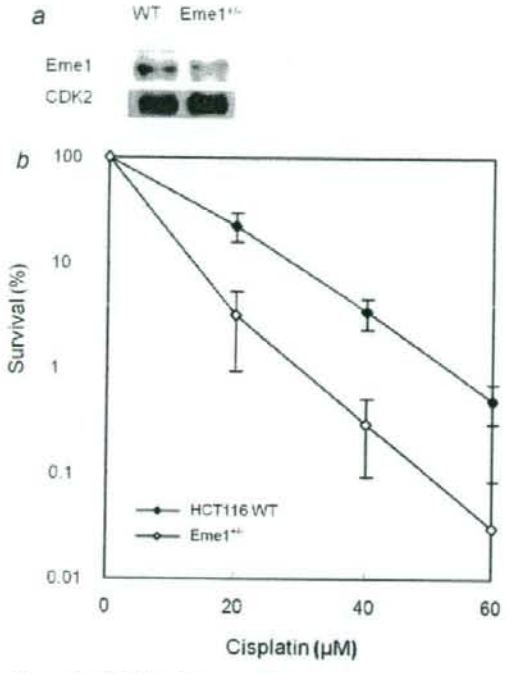

FIGURE 1 – Haploinsufficiency of *Eme1* leads to hypersensitivity to cisplatin in HCT116 cells. (*a*) Western blot analysis confirming Eme1 levels. Western blotting for CDK2 was carried out to confirm equal loading. (*b*) Sensitivity to cisplatin in HCT116 cells. Values represent means ± standard error for 3 independent experiments.

Northern blot analysis

Poly(A)$^+$ RNAs were isolated using PolyATtract mRNA isolation systems (Promega). Hybridization was performed overnight at 65°C in hybridization buffer (0.5 M $NaPO_4$ pH 7.2, 1 mM EDTA, 7% SDS and 1% bovine serum albumin), followed by washing 3 times for 30 min at 65°C in washing buffer (40 mM $NaPO_4$ pH 7.2, 1 mM EDTA and 1% SDS). Probes were amplified by PCR from cDNA derived from normal human cells. The full-length Eme1 cDNA was amplified using primers 5′-AGTTGAAA GAGTGGCGGGA-3′ and 5′-CTCATCCCTGAGGGCTAGAA-3′. The glyceraldehydes 3-phosphate dehydrogenase (GAPDH) fragment was amplified using primers 5′-ACCACAGTCCATGC CATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. BAS-2500 (FUJIFILM) was used for quantitative analysis.

Sensitivity to cisplatin

Cells were treated with cisplatin for 1 hr, washed with phosphate-buffered saline 3 times and plated at a density of 2, 4 or 6 × 10^3 cells per 60 mm dish. The numbers of colonies were counted after 7 to 16 days of culture. *D*37 values were determined from a least squares regression fit to the linear portion of the dose-response curve.

Results and discussion

Eme1 haploinsufficiency leads to hypersensitivity to cisplatin in HCT116 cells

We previously showed increased sensitivity to MMC in *Eme1*$^{+/-}$ HCT116 cells.[14] To investigate Eme1's role in cisplatin sensitivity, the same cells were treated with the drug. Western blot analysis revealed that the Eme1 level in *Eme1*$^{+/-}$ cells was about half the wild-type level (Fig. 1*a*). The mutant cells exhibited mild hypersensitivity to cisplatin (2-fold), suggesting that Eme1 is involved in the regulation of cisplatin sensitivity (Fig. 1*b*). This observation is consistent with the role of the Mus81-Eme1 endonuclease complex in the repair of ICL.

Levels of proteins involved in the repair of ICL in human cancer cell lines

To investigate Eme1's role in the regulation of cisplatin sensitivity, levels of the protein were examined in 18 human tumor cell lines. The levels in RPE and HME cells were defined as moderate. Only 6 tumors exhibited moderate Eme1 levels, indicating that the levels varied among cell lines. High Eme1 levels were found in 7

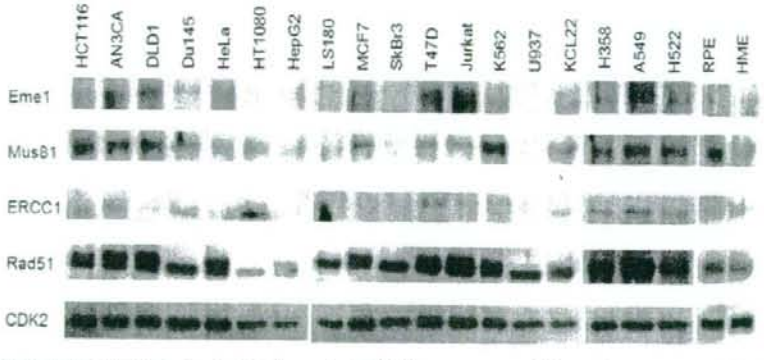

FIGURE 2 – Expression levels of proteins involved in the repair of ICL in human cancer cell lines. Western blot analysis shows the expression of Eme1, Mus81, ERCC1 and Rad51.

TABLE I – EXPRESSION LEVELS OF PROTEINS INVOLVED IN THE REPAIR OF ICL AND *D37* VALUES OF SENSITIVITY TO CISPLATIN IN HUMAN CANCER CELL LINES

Cell line	Origin	Eme1	Mus81	ERCC1	Rad51	D37 (μM)
HCT116	Colorectal carcinoma	→	→	→	↑	16.6
AN3CA	Endometrial carcinoma	↑	→	→	↑	
DLD1	Colorectal carcinoma	↑	↑	↓	↑	48.2
Du145	Prostatic carcinoma	→	→	→	→	
HeLa	Cervical carcinoma	↑	→	↓	↑	29.9
HT1080	Fibrosarcoma	↓	→	→	↓	18.5
HepG2	Hepatocellular carcinoma	↓	↓	↓	↓	
LS180	Colorectal carcinoma	↓	↓	↓	→	17.0
MCF7	Breast carcinoma	↑	→	→	↑	
SkBr3	Breast carcinoma	↓	↓	↓	→	12.9
T47D	Breast carcinoma	↑	→	→	↑	43.1
Jurkat	Acute T cell leukemia	↑	→	→	↑	
K562	Chronic myelogenous leukemia	→	↑	→	↑	
U937	Histiocytic lymphoma	↓	↓	↓	→	
KCL22	Chronic myelogenous leukemia	→	→	↓	↑	
H358	Lung carcinoma	→	→	→	↑	15.0
A549	Lung carcinoma	↑	↑	→	↑	31.6
H522	Lung carcinoma	→	→	→	→	13.6

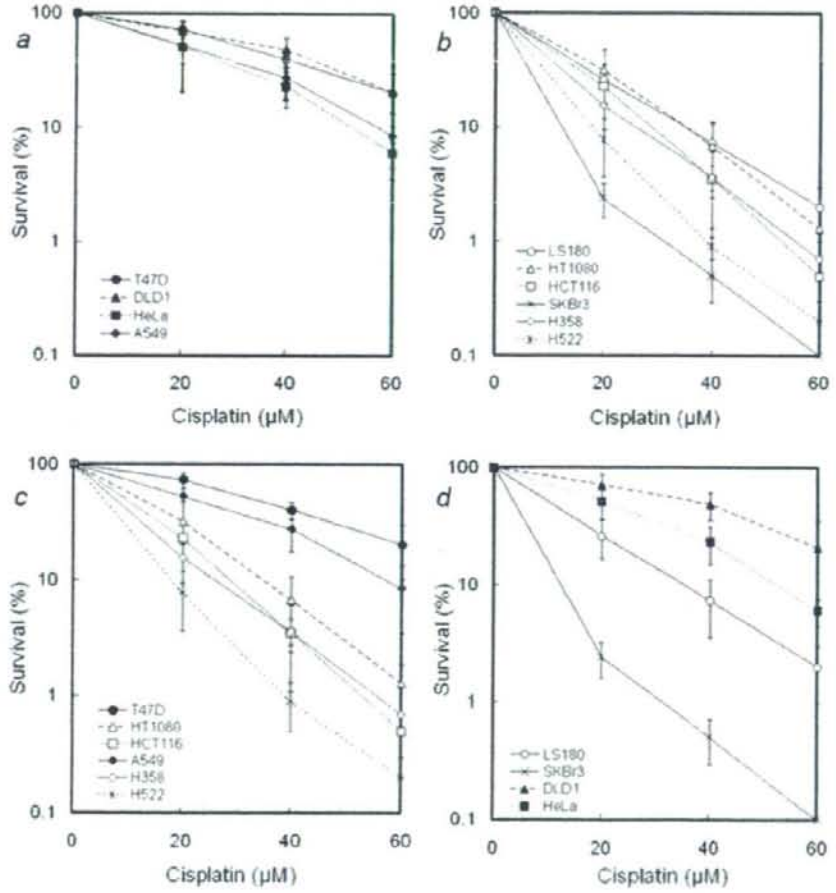

FIGURE 3 – Eme1 levels correlate well with cisplatin sensitivity. Values represent means ± standard error for 3 independent experiments. (*a*) Cisplatin sensitivity in cells with high Eme1 levels. (*b*) Cisplatin sensitivity in cells with moderate or low Eme1 levels. (*c*) Cisplatin sensitivity in cells with moderate ERCC1 levels. (*d*) Cisplatin sensitivity in cells with low ERCC1 levels.

cell lines and low levels in 5 cell lines (Fig. 2). We also examined whether Emel protein levels were affected by cisplatin treatment. Emel protein levels were not changed after cisplatin administration in DLD1 cells, indicating that cisplatin does not affect Emel expression levels (Supp. Info. Fig. 1).

Because, Emel forms a heterodimeric complex with Mus81, Emel levels are likely to correlate with Mus81 levels. Clear correlations were observed in 11 cell lines and less clear correlations were seen in the other 7 lines. It is of interest that the expression of both Emel and Mus81 was lost in U937 cells. Moreover, both were barely expressed in HepG2, LS180 and SkBr3 cells.

ERCC1 has been extensively studied as a marker of cisplatin sensitivity.[6–8] To investigate the feasibility of Emel levels serving as a marker, it is very important to compare the levels of Emel with those of ERCC1. Moderate ERCC1 levels were observed in 11 cell lines, but the levels were low in the other 7 lines (Fig. 2). In 8 cell lines, ERCC1 levels were correlated with both Mus81 and Emel levels (Table I). However, a negative or no correlation was observed in several tumors. In DLD1 cells, ERCC1 expression was hardly detectable, whereas Emel and Mus81 were highly expressed. In HeLa cells, ERCC1 expression was low, whereas that of Emel was high. In HT1080 cells, ERCC1 and Mus81 levels were moderate and that of Emel was low. In AN3CA, MCF7, T47D and Jurkat cells, Emel levels were high and ERCC1 and Mus81 levels were moderate. In K562 cells, ERCC1 and Emel were moderate and Mus81 was high. In KCL22 cells, ERCC1 was low whereas Emel and Mus81 were moderate. In A549 cells, Emel and Mus81 were high and ERCC1 was moderate. Thus, in more than half of the cell lines examined, the expression levels of the 3 proteins were not correlated.

In addition to ERCC1, Rad51 has been investigated as a marker of cisplatin sensitivity.[9] Unlike the other 3 proteins, Rad51 was overexpressed in 11 cell lines and only 2 samples showed low expression. There was no apparent correlation between Rad51 and any of the other proteins.

Emel protein levels correlate well with cisplatin sensitivity

To investigate the relationship between Emel and ERCC1 levels and cisplatin sensitivity, we chose 10 cell lines based on the levels of these proteins and examined cell survival after treatment with the drug (Fig. 3 and Table I). Reduced sensitivity to cisplatin represented by high $D37$ values was observed in DLD1 ($D37$ = 48.2 μM), T47D (43.1 μM), HeLa (29.9 μM) and A549 (31.6 μM) cells. Moderate hypersensitivity was observed in HT1080 (18.5 μM), LS180 (17.0 μM), HCT116 (16.6 μM) and H358 (15.0 μM) cells. Remarkable hypersensitivity was observed in SkBr3 (12.9 μM) and H522 (13.6 μM) cells.

Emel levels in tumors with reduced sensitivity to cisplatin (T47D, DLD1, HeLa and A549) were high, whereas the levels in tumors with increased sensitivity (HT1080, LS180, HCT116, SkBr3, H358 and H522) were moderate or low (Figs. 3*a* and 3*b*). These results indicate that Emel levels correlate well with cisplatin resistance. Good correlation was also observed with Mus81. However, T47D and HeLa cells were exceptions, as they were resistant to cisplatin with no increase in Mus81 levels. In addition, we examined Emel mRNA levels by Northern blot analysis. In HCT116, HT1080, LS180, SkBr3 and T47D cells, Emel mRNA levels correlated with the protein levels (Supp. Info. Fig. 2). However, a negative or no correlation was observed in other tumors. In DLD1 cells, the protein level was high, whereas the mRNA level was low. In H358 and H522 cells, the proteins levels were moderate, whereas the mRNA levels were high. These findings suggest that the Emel levels are regulated at post-transcriptional levels in some tumors. Similarly, no obvious relationship between ERCC1 protein levels and mRNA levels was reported.[15]

In tumors with moderate ERCC1 levels, cisplatin resistance was observed in T47D cells but not in HT1080 or HCT116 cells (Fig. 3*c*). In tumors with low ERCC1 levels, hypersensitivity to cisplatin was observed in SkBr3 and LS180 cells but not in DLD1 or HeLa cells (Fig. 3*d*). This observation indicates that the correlation between ERCC1 and cisplatin sensitivity was not so clear. Tumors with high Rad51 levels were resistant to cisplatin, except for HCT116 and H358 cells, which were moderately sensitive. Tumors with moderate or low Rad51 levels (LS180, SkBr3, HT1080 and H522) were hypersensitive to cisplatin.

These observations indicate that cisplatin sensitivity correlated well with Emel levels and to a lesser extent with Rad51 and Mus81 levels. The Mus81-Emel endonuclease complex plays a role in the repair of ICL upstream of the XPF-ERCC1 complex.[10] This epistasis may explain the superiority of Emel and Mus81 as markers of cisplatin sensitivity. Alternatively, other proteins might complement the reduced ERCC1 activity levels observed in tumors with cisplatin resistance.

ERCC1 levels were altered in primary tumor samples, including NSCLC, colorectal, gastric and ovarian cancers.[16] Also, Rad51 levels were altered in primary tumor samples including NSCLC, pancreatic, colorectal, breast, head and neck cancers.[16] Given that Emel and Mus81 levels are altered in primary tumors, it is highly likely that these proteins can be useful as markers of cisplatin resistance. It is also possible that the measurement of a combination of the 4 proteins examined here may predict cisplatin sensitivity more accurately than that of each protein alone.

These results may also lead to the development of potential therapeutic agents, such as antibodies and small molecules that inhibit the ICL repair proteins. Cisplatin has been shown to be effective against many types of cancers. All too often, however, initially responsive tumors recur, usually without sensitivity to the drug. Such acquired resistance to the drug is a major obstacle to curing cancers with cisplatin.[1] The identification of Emel as a marker of cisplatin resistance will be useful for the development of resistance modulators or new molecularly targeted drugs.

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