INTRODUCTION

In the developing brain, multipotent neural progenitor cells (NPCs) proliferate in the ventricular zone (VZ) (Rao, 1999; Qian et al., 2000; Temple, 2001). In the early developmental stage, they form a pseudostratified epithelium in the VZ, so they also are called neuroepithelial cells. Later, they project their fibers from the ventricular surface to the pial surface, where they are called radial glia. As mentioned later in detail, the nuclei of proliferating NPCs undergo a characteristic migration in the VZ (Takahashi et al., 1995; Fujita, 2003).

Since a large number of processes such as proliferation, migration, and differentiation of neural cells occur during an extended period of development, the fetal central nervous system (CNS) is sensitive to diverse environmental factors including DNA-damaging agents. This paper reviews the mechanisms of neurotoxicity induced in the developing brain of mice and rats by six chemicals (ethylnitrosourea, hydroxyurea, 5-azacytidine, cytosine arabinoside, 6-mercaptopurine and etoposide), which cause DNA damage in different ways, especially from the viewpoints of apoptosis and cell cycle arrest in neural progenitor cells. In addition, this paper also reviews the repair process following damage in the developing brain.

The balance between cell proliferation and cell death (apoptosis) is important for correct development of the brain (Oppenheim, 1991; Blaschke et al., 1996; Kuida et al., 1996; Thomaidou et al., 1997). Moreover, the regulation of this balance also seems to be important during damage due to extrinsic stresses. Cytotoxic stresses induce excessive cell death and suppress cell proliferation in the developing brain (Katayama et al., 2002, 2005a; Ueno et al., 2002a, 2002b; Yamauchi et al., 2003; Sehata et al., 2004; Semont et al., 2004; Nam et al., 2006a, 2006b; Woo et al., 2006; Kanemitsu et al., 2009a), and such excess cell death by apoptosis may bring about a lack of cell populations required for later normal histogenesis and organogenesis, resulting in anomalies in the offspring (Inouye and Murakami, 1978; Ferrer et al., 1982, 1984; Miki et al., 1995; Zhang et al., 1995; Sun et al., 1996, 2001; Fushiki et al., 1997; Katayama et al., 2000a, 2000b; Kitamura et al., 2001; Woo et al., 2004; Furukawa et al., 2007). Elucidating the mechanisms of how NPCs regulate the cell cycle and apoptosis and respond to exogenous stimuli is important for understanding both normal and abnormal development of the CNS. However, it is not widely known how the developing brain responds to extrinsic damage. Moreover, it is even less well known how the repair or recovery process following damage in the developing brain is regulated (Oyanagi et al., 1998; Kikuchi-Horie et al., 2004; Ueno et al., 2006c).
This paper reviews the mechanisms of neurotoxicity induced in the developing brain of mice and rats by six chemicals (ethylnitrosourea, ENU; hydroxyurea, HU; 5-azacytidine, 5AzC; cytosine arabinoside, Ara-C; 6-mercaptopurine, 6-MP; etoposide, VP-16), which cause DNA damage in different ways, especially from the viewpoints of apoptosis and cell cycle arrest in NPCs. This paper also reviews the repair process following damage in the developing brain.

NUCLEAR MIGRATION AND CELL CYCLE PROGRESSION OF NPC

As mentioned above, the nuclei of proliferating NPCs undergo a characteristic migration — interkinetic nuclear migration (or elevator movement) — in the VZ, in which the positions of the nuclei are correlated with their cell cycle phase (Sauer and Walker, 1959; Sidman et al., 1959; Fujita, 1962; Bayer and Altman, 1995; Takahashi et al., 1993, 1995; Hayes and Nowakowski, 2000). In brief, S-phase nuclei, located in the outer area of the VZ, migrate inward during the G2 phase, and mitosis occurs at the ventricular surface. The nuclei then migrate outward during the G1 phase and enter the S phase again (Fig. 1). In this way, NPCs proliferate. After mitosis, some of them migrate beyond the VZ, where they differentiate into neurons or glial cells to form the neural network, e.g., cortex in the telencephalon (Tamamaki et al., 2001; Temple, 2001; Anthony et al., 2004; Miyata et al., 2004; Noctor et al., 2004). This region is generally called the marginal zone or cortical plate in the developing stage of telencephalon.

Migration and translocation of NPCs are important processes for correct brain development, and this migration is necessary to segregate the VZ into two regions, the inner area and the outer area, to maintain neurogenesis and DNA replication, respectively (Murciano et al., 2002). Thus, this migration seems to be important for completing the brain development in which neurons and glial cells have to be produced in a large number and with an appropriate timing.

NPCs seem to be necessary to proliferate in a precise regulation of cell cycle and nuclear migration. Ueno et al. (2006b) examined whether this nuclear migration pattern correlates with cell cycle progression even when the cell cycle progression is chemically disrupted. As a result, NPCs stopped their migration according to their phase of cell cycle arrest. For example, 5AzC-treatment induced M or G2 arrest, and the nuclei of NPCs stopped their migration along the ventricles and in the inner area of the VZ, where they would normally be located during the M and G2 phase, respectively. In addition, ENU- or cyclophosphamide-treatment induced S-phase arrest, and the nuclei of NPCs stopped their migration in the outer area of the VZ, where the nuclei of S-phase cells normally exist (Katayama et al., 2005a; Ueno et al., 2006b). Moreover, chemically induced mitotic arrest to the NPCs stopped their migration at the ventricular surface (Kallen, 1961, 1962; Langman et al., 1966). On the other hand, inhibition of nuclear migration did not affect cell cycle progression (Karfunkel, 1972; Messier and Auclair, 1974; Webster and Langman, 1978; Murciano et al., 2002; Gambello et al., 2003). These findings suggest that cell cycle regulators control the migration, but the migration system does not control cell cycle. In this context, Ueno et al. (2006b) clarified that nuclear position within the VZ is correlated with cell cycle phase even when the cell cycle is disrupted, and that the nuclei of NPCs can migrate only when their cell cycle is going. They suggested that cell cycle regulators might control the machinery of migration through a common regulatory mechanism.

There are several reports of genes which are considered to be related to migration of NPCs, and these reports show that some genes play a role in the signal transduction through the plasma membrane (Ohshima and Mikoshiba, 2002) while other genes are thought to directly regulate the cytoskeleton and mediate cell movement (Feng and Walsh, 2001). In addition, it is suggested that Ca2+, gap-junctional communication, or hemichannels...
may be involved in cell cycle progression and nuclear migration (Owens and Kriegstein, 1998; Becker and Mobbs, 1999; Cheng et al., 2004; Weissman et al., 2004).

**APOPTOSIS AND CELL CYCLE ARREST IN NPC**

Studies on fetal neurotoxicity in mice and rats have generally been carried out by exposure of dams to DNA-damaging agents on day 12 or 13 of gestation (GD 12 or 13) when the developing brain seems to be highly sensitive to such agents (Pfaffenroth et al., 1974; Hallas and Das, 1978; D’Sa-Eipper et al., 2001). The nature of fetal neurotoxicity induced by DNA-damaging chemicals is similar between rats and mice (Katayama et al., 2005a; Ueno et al., 2006a, 2006b).

As mentioned above, cytotoxic stresses including DNA damage induce excess apoptotic cell death and suppress cell proliferation in the developing brain of mice and rats. In the VZ of telencephalon, apoptotic cells are mainly observed in the dorsal layer after treatment with ENU (Katayama et al., 2000a) and Ara-C (Yamauchi et al., 2003), in the medial to dorsal layers after treatment with HU (Woo et al., 2003), 6-MP (Kanemitsu et al., 2009c) and VP-16 (Nam et al., 2006a), and in the ventral to medial layers after treatment with 5AzC (Ueno et al., 2002b). As shown in Fig. 2, the number of apoptotic NPCs peaks at 9 to 12 hr and returns to the control level at 48 hr after treatment with ENU (Katayama et al., 2001), HU (Woo et al., 2006), 5AzC (Ueno et al., 2002b), Ara-C (Yamauchi et al., 2004a) and VP-16 (Nam et al., 2006a), while it peaks at 30 hr and returns to the control level at 96 hr after treatment with 6-MP (Kanemitsu et al., 2009c). Following radiation, it peaks at 3 to 5 hr (Borovitskaya et al., 1996).

The sequence of the number of mitotic NPCs is more variable among DNA-damaging chemicals (Fig. 3). The number of mitotic NPCs decreases in accordance with the increase in the number of apoptotic NPCs, and the numbers of both mitotic and apoptotic NPCs almost simultaneously return to the control levels following the treatment with ENU (Katayama et al., 2001), Ara-C (Yamauchi et al., 2004a) and 6-MP (Kanemitsu et al., 2009c). After VP-16 treatment (Nam et al., 2006a), the number of mitotic NPCs reaches the minimum level at 4 hr and returns to the control level at 8 hr. The number of mitotic NPCs reaches the maximum level at 12 hr and returns to the control level at 24 hr after HU administration (Woo et al., 2006). After treatment with 5AzC (Ueno et al., 2002b), the number of mitotic NPCs peaks earlier than the number of apoptotic NPCs at 6 hr, decreases thereafter, reaches the minimum level at 24 hr, and then returns to the control level at 48 hr. At 6 hr, many mitotic cells accumulate in the VZ facing the lateral ventricle, and some of them show pleomorphic mitotic figures as observed in tumor cells (Timonen and Therman, 1950; Henderson and Papadimitriou, 1982). Ueno et al. (2002b) suggested that 5AzC might induce defective mitotic synchronization in NPCs in the developing brain.

The above-mentioned differences in the location of apoptotic cells in the VZ and the time-course changes of apoptotic and mitotic NPCs seem to reflect the differences in pharmacokinetics, mechanisms of DNA damage, signaling pathways involved in apoptosis and phases of cell division.
cycle arrest among DNA-damaging chemicals (Katayama et al., 2001; Yamauchi et al., 2004a).

It is reported that ataxia-telangiectasia mutated (ATM) protein is activated in response to DNA damage, and the activated ATM protein induces phosphorylation of the p53 protein, which protects itself from destruction by its negative regulator, mdm2 (Shie et al., 1997; Banin et al., 1998; Canman et al., 1998; Lee et al., 2001; Nam et al., 2010). In a lot of cases, the activity of p53 is known to be mediated by such a post-transcriptional protein stabilization mechanism.

DNA damage induces cell cycle arrest and apoptosis via ATM-checkpoint kinase 2 (Chk2) responses (DiTullio et al., 2002; Ha et al., 2003) as reported in the cellular response to irradiation and topoisomerase inhibitors (Samuel et al., 2002). Moreover, DNA damage-dependent focal accumulation of both DNA repair factors and γH2AX is observed in the nucleus, i.e., so-called repair foci. H2AX phosphorylation has an important role in the initiation of DNA repair (Downs et al., 2000). To date, however, there are only a few reports directly showing the activation of ATM in the developing brain after treatment with DNA-damaging agents (Nam et al., 2010).

p53 is known as a “guardian of genome”, and plays a critical role in DNA repair, cell cycle arrest, and apoptosis in response to DNA damage (Morrison et al., 1996; Uberti et al., 1998; Lakin and Jackson, 1999; Katayama et al., 2001). Activated p53 increases the expression of its transcriptional target genes such as p21, bax, cyclinG1, fas and gadd45 (Borovitskaya et al., 1996; Prives and Hall, 1999; Bolaris et al., 2001). p21 is an inhibitor of cyclin-dependent kinases and it induces cell cycle arrest at G1 phase (El-Deiry et al., 1993; Dulic et al., 1994). Bax is a pro-apoptotic member of the bcl-2 family (Selvakumaran et al., 1994), and the Bax/Bcl-2 ratio enforces dimerization of Bax, which finally induces apoptosis through the intrinsic pathway (Miyashita and Reed, 1995; Gross et al., 1998). Bax accelerates the release of the apoptosis-inducing factor (AIF) and cytochrome c from the mitochondria, thus activating the caspase cascade (Sionov and Haupt, 1999). Fas is a type I membrane protein which belongs to the tumor necrosis factor receptor/nerve growth factor receptor family (Itoh et al., 1991), and it induces apoptosis through the extrinsic pathway when it binds to Fas ligand (Nagata and Golstein, 1995). Cyclin G1 is thought to be involved in DNA replication because it localizes in the replication foci at S phase (Reimer et al., 1999). Gadd45 is also known to play an important role in DNA repair (Smith et al., 1994).

Ethynitrosourea (ENU)

ENU is a well known DNA alkylating agent with high mutagenicity (Shibuya and Morimoto, 1993). ENU is also known as a selective neurogenic carcinogen (Koestner, 1990; Vaquero et al., 1994) and as a teratogen (Pfaffneroth et al., 1974; Ohnishi, 1989; Katayama et al., 2000a).

Katayama et al. (2005a) demonstrated by flow cytometric analysis that an accumulation of NPCs in the S phase occurred immediately after the administration of ENU on GD13. They also showed by BrdU-incorporation assay that most of these NPCs contained DNA identical to that of S-phase cells but did not incorporate BrdU. Soon after this accumulation reached its peak, the number of cells in the G2/S phase decreased and the apoptotic cell count increased. In addition, prior to the occurrence of ENU-induced apoptosis and cell cycle arrest, the expression of p53 protein increased, and transcription of its target genes such as p21, bax, cyclinG1 and fas was also activated (Katayama et al., 2002). As mentioned above, it is well known that p21 is transactivated by p53 and induces cell cycle arrest at the G1 phase. However, the expression of cyclin D1 and CDK4, the cell cycle machinery of the G1/S transition, was not up-regulated in Western blot analysis, and the number of cells in the G0/G1 phase decreased and that in the S phase increased on administration of ENU (Katayama et al., 2002). These findings indicate that the cell cycle is arrested in the S phase rather than in the G1 phase, and that ENU inhibits or arrests DNA replication in NPCs during the S phase and then evokes apoptosis before the cells enter the G2 phase (Fig. 4). Thus, the precise role of p21 in ENU-induced

Fig. 4. Mechanisms of ENU-induced neurotoxicity in the fetal rat brain. ENU induces inhibition of DNA replication in NPCs during S phase and then evokes apoptosis before the cells enter G2 phase. p53 is required for both ENU-induced apoptosis and cell cycle perturbation in S phase. CP: cortical plate; VZ: ventricular zone; NPC: neural progenitor cell.
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fetal neurotoxicity is not clear. In addition, the number of p21-positive NPCs after ENU administration was much less than that of p53-positive cells (Katayama et al., 2002). Therefore, the fetal CNS exposed to ENU would tend to eliminate DNA-damaged cells via apoptosis rather than arrest the cell cycle and repair the DNA lesions.

In p53-deficient mice, both ENU-induced apoptosis and S-phase accumulation were almost completely abrogated, indicating that both ENU-induced apoptosis and cell cycle perturbation in the S phase require p53 and that ENU may induce apoptosis of NPCs through the p53-mediated intrinsic pathway (Katayama et al., 2005a) (Fig. 4). However, the mechanism of the p53-dependent induction of S-phase accumulation in the fetal rat brain exposed to ENU is still obscure as previously pointed out in fetal rat cells exposed to 5-fluorouracil, a thymidine synthase inhibitor (Elstein et al., 1993; Shuey et al., 1994). On the other hand, in C57BL/6J-prl/prl mice, which lack Fas/CD95 (Watanabe-Fukunaga et al., 1992), apoptosis and cell cycle perturbation were also observed upon administration of ENU, as in the wild-type C57BL/6J mice, indicating that Fas/CD95 is not critical for ENU-induced apoptosis or cell cycle alteration in the fetal CNS.

It is well known that ENU alkylates mainly the O6 position of guanine (Shibuya and Morimoto, 1993), and the existence of alkyl lesions in the genomic DNA inhibits DNA replication (Ceccotti et al., 1993; Eckert et al., 1997). The brain eliminates O6-alkylguanine at a much lower rate than other organs, and long-term retention of O6-alkylguanine in the brain is thought to be the cause of brain neoplasm (Koestner, 1990). In addition, ENU has little induction activity of gadd45 in the fetal brain (Katayama et al., 2002). This brings about the remainder of the DNA damage in the fetal brain, and finally results in the high incidence of brain neoplasms after birth. Therefore, elimination of DNA-damaged cells by p53-dependent apoptosis is very important for the prevention of brain tumorigenesis (Leonard et al., 2001).

Hydroxyurea (HU)

HU directly inhibits DNA synthesis as a result of its inhibiting effect on ribonucleotide reductase without affecting RNA or protein synthesis (Yarbro et al., 1965), and HU has been used as a chemotherapeutic agent for various types of cancer, HIV infection and sickle cell anemia (Woo et al., 2003). On the other hand, HU is also known as a teratogen (Barr and Beaudoin, 1981; Woo et al., 2004).

Woo et al. (2006) reported that the expression of p53 protein phosphorylated at serine 15 and 20 was strongly elevated prior to the peak of apoptosis in the fetal mouse brain after treatment with HU on GD13. The expression of p53-target genes (fas, fasL and bax and p21) was also significantly elevated at the mRNA level by RT-PCR analysis and at the protein level by Western blot analysis. Mdm protein began to increase at 12 hr when the expression level of p53 began to reduce. Flow-cytometric and Western blot analyses of cell cycle-related proteins revealed that NPCs were arrested at the S checkpoint from 3 to 6 hr and at the G2/M checkpoint at 12 hr, respectively, resulting in apoptosis. p53 and p21 are necessary to maintain G2 arrest following DNA damage (Flatt et al., 2000) and the mechanism of p53-dependent G2 arrest involves an initial inhibition of cyclin B1/cdc2 activation by p21 and subsequent reduction of cyclin B1 and cdc2 protein levels (Innocente et al., 1999; Flatt et al., 2000). Woo et al. (2006) suggested that HU-induced apoptosis in NPCs may be mediated by p53 and caused through both intrinsic (mitochondria-related) and extrinsic (fas/fasL-related) pathways.

5-Azacytidine (5AzC)

5AzC is a cytidine analogue which possesses a nitrogen atom instead of a carbon atom at the 5 position of the pyrimidine ring (Sorm et al., 1964), and it is now used for the chemotherapy of acute myeloid leukemia and myelodysplastic syndrome (Pinto and Zagonel, 1993; Cheson et al., 2000).

5AzC may disturb gene expression and alter organogenesis through its DNA demethylating effect (Bender et al., 1998; Egger et al., 2004; Claus et al., 2005). 5AzC also acts as DNA-damaging agent. Namely, when 5AzC is incorporated into DNA, it forms a covalent bond with DNA methyltransferase (Santi et al., 1984; Michalowsky and Jones, 1987; Ferguson et al., 1997), resulting in DNA damage (Juttermann et al., 1994; Karpf et al., 2001). Ueno et al. (2002a, 2006a) showed that any gene whose expression is up-regulated by the demethylating effect was not identified in DNA microarray analysis on the fetal rat brain exposed to 5AzC on GD13. They also showed that p53 expression was augmented at the protein level in NPCs and the expression of some target genes of p53 was up-regulated. Therefore, they surmised that 5AzC exerts a toxic effect via DNA damage in the developing brain as previously proposed by Juttermann et al. (1994).

5AzC first induces the accumulation of cells in abnormal mitosis, G2 phase accumulation, and then apoptosis of NPCs (Ueno et al., 2006a). Most of the apoptotic cells are in G1 phase. Cell cycle transition studies suggest that G2/M progression (firstly M and secondly G2 progression) is blocked, after which the cells move to G1.
phase or undergo apoptosis (Ueno et al., 2006a). Namely, some cells pass from G2/M to G1 phase with completion of correct DNA repair, others enter G1 but undergo apoptosis because of incomplete repair, and still others stay in G2/M to undergo apoptotic cell death. The cells dying at G1 phase after mitosis might have died due to mitotic catastrophe, as a result of incomplete mitosis and faulty DNA repair at G2 or S phase (Fig. 5). Castedo et al. (2004) reported that aberrant entry into mitosis after DNA damage, gene deficiencies, or excess activation of cdc2-cyclin B1 each suffices to cause mitotic catastrophe.

p53 and some of its target genes (p21, cyclin G1, Igfhp3, Mdm2 and Snk) showed increased expression in Western blot and DNA microarray analyses in the fetal brain after 5AzC treatment. On the other hand, in 5AzC-treated fetal brains of p53-deficient mice, apoptosis did not occur, although G2/M accumulation was induced (Ueno et al., 2006a). This suggests that apoptosis is p53-dependent but another mechanism governs the G2/M checkpoint in the developing brain after treatment with 5AzC (Fig. 5).

At the G2/M checkpoint, the normal cell cycle transition from G2 to M is dependent on Cyclin B1-Cdc2 complex, which is activated by Cdc25. DNA damage generally activates Chk2 (Chaturvedi et al., 1999; Liu et al., 2000; Matsuoka et al., 2000) and inhibits Cdc25 function by phosphorylation, and then leads to G2 arrest through inactivation of Cdc2 (Hunter, 1995; Watanabe et al., 1995). The Cyclin B1-Cdc2 complex becomes inactive after phosphorylation of tyrosine 15 and threonine 14 of Cdc2. However, in the case of 5AzC-induced DNA damage in the fetal brain, Ueno et al. (2006a) demonstrated by microarray, Western blot and signal pathway analyses that phospho-Cdc2 decreased from 6 to 12 hr although G2 progression was blocked with delay in inward-migration. This abnormal activation of Cdc2 might accelerate the G2 to M transition, probably resulting in an accumulation of cells showing abnormal mitosis (Fig. 6). Furthermore, the expression of cyclin B1 was slightly increased from 3 to 9 h after treatment with 5AzC. Cyclin B1 is degraded by anaphase-promoting complex (APC) at metaphase, and this process is indispensable for mitotic progression (Pines and Hunter, 1991), so the accumulation of cyclin B1 observed after 5AzC treatment might occur as a result of inactivation of APC, which leads to mitotic arrest (Nitta et al., 2004).

**Cytosine arabinoside (Ara-C)**

Ara-C, a cytidine analogue, exerts its cytotoxic effect through the inhibition of DNA synthesis, i.e., the inhibition of replicative DNA polymerases, when it is incorporated into DNA in S phase and causes DNA damage. Damaged NPCs enter M phase due to abnormal regulation at G2/M checkpoint, resulting in accumulation of abnormal mitotic NPCs along the ventricle. Then, the cells are divided into daughter cells but undergo apoptosis in G1 phase through mitotic catastrophe. G2-phase arrest also occurs with delay in inward-migration, and G2-phase cells also die by apoptosis. Apoptosis is p53-dependent while abnormal G2/M regulation is p53-independent. CP: cortical plate; VZ: ventricular zone. By courtesy of Dr. Masaki Ueno (Ph.D. Thesis of the University of Tokyo, 2006, pp.70).

**Fig. 5.** Mechanisms of 5AzC-induced neurotoxicity in the fetal rat brain. 5AzC would be incorporated into DNA in S phase and cause DNA damage. Damaged NPCs enter M phase due to abnormal regulation at G2/M checkpoint, resulting in accumulation of abnormal mitotic NPCs along the ventricle. Then, the cells are divided into daughter cells but undergo apoptosis in G1 phase through mitotic catastrophe. G2-phase arrest also occurs with delay in inward-migration, and G2-phase cells also die by apoptosis. Apoptosis is p53-dependent while abnormal G2/M regulation is p53-independent.

**Fig. 6.** G2/M checkpoint. Normal cell cycle transition from G2 to M is dependent on Cyclin B1-Cdc2 complex. Following DNA damage, Cdc2 of the Cyclin B1-Cdc2 complex is generally phosphorylated (inactivated), resulting in prevention of G2/M transition. However, following 5AzC-induced DNA damage, abnormal activation (dephosphorylation) of Cdc2 occurs, and this accelerates entry into M phase, leading to an accumulation of abnormal mitotic cells.
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rated into the end of growing DNA strand (Woodcock, 1987). Thus, Ara-C seems to affect mainly the S-phase cells (Grant, 1998). Ara-C has been used as one of the most effective agents in the clinical treatment for myelogenous leukemia (Capizzi, 1996). On the other hand, prenatal treatment with Ara-C has a teratogenic effect and causes malformations of the brain in rodents (Chaubé et al., 1968; Adlard et al., 1975; Percy, 1975).

Yamauchi et al. (2004a) examined the neurotoxic effects of Ara-C in the fetal rat brain. Ara-C induced cell cycle arrest and apoptosis of NPCs in the S phase when administered to dams on GD13. In their study, prior to the induction of apoptosis, the expression of p53 protein was elevated markedly and p21 showed intense over-expression at protein and mRNA levels following the increase in p53 protein. RT-PCR analysis revealed that the mRNA expressions of other p53 transcriptional target genes, bax, cyclinG1 and fas, also increased in concurrence with the increase in apoptotic cells. In addition, D’Sa-Eipper et al. (2001) reported that in p53–deficient mice treated with Ara-C, apoptosis was completely abrogated while Ara-C-induced cell cycle arrest was not altered in the fetal brain. Therefore, they suggest that Ara-C induces apoptosis of NPCs through the p53-mediated intrinsic pathway while cell cycle arrest is p53-independent. Although they did not check the changes in the induction of Ara-C-induced apoptosis of NPCs in Fas-mutated lpr/lpr mice, Yamauchi et al. (2007) reported that the incidence of Ara-C-induced trophoblastic apoptosis in the placenta was similar between lpr/lpr and wild-type mice, suggesting that Ara-C-induced NPC apoptosis may also be Fas-independent.

Yamauchi et al. (2004a) also reported that in the fetal brain exposed to Ara-C, small secondary peaks were observed in the expression patterns of p53 and p21 proteins at 24 hr after treatment. They proposed two possible explanations for this finding. First, NPCs in the telencephalic VZ are not composed of homogeneous cells and reactions to Ara-C treatment would be different among these cells. Second, maternal and especially placental toxicity of Ara-C (Yamauchi et al., 2004b) would affect the fetal tissues including the brain through hypoxia. Hypoxia is known to cause p53 protein accumulation and over-expression of its downstream targets in cultured neurons (Bossemeyer-Pourie et al., 2002).

6-Mercaptopurine (6-MP)

6-MP, an analogue of hydroxanthine, is widely used for the therapy of acute lymphoblastic leukemia and as an immunosuppressive drug (Estlin, 2001; Nielsen et al., 2001). 6-MP is first metabolized to the active form within the cell (Elion, 1967). This active metabolite inhibits the activities of enzymes in the de novo purine synthetic pathway (Atkinson et al., 1963; Bridger and Cohen, 1963; McCollister et al., 1964). It is further converted to thioguanine, which is incorporated into nucleic acid and induces cytotoxicity and fetotoxicity (Tidd and Paterson, 1974; Adhami and Noack, 1975; Hoki, 1990).

In the study of Kanemitsu et al. (2009b) on the neurotoxicity of 6-MP in the fetal rat brain, flow-cytometric analysis confirmed an accumulation of NPCs at G2/M from 24 to 36 hr, S from 36 to 48 hr and sub-G1 (apoptotic cells) phases from 36 to 48 hr after treatment with 6-MP on GD13. In addition, the phosphorylated (active) form of p53 protein increased from 24 to 48 hr, and p21 simultaneously showed intense over-expression at both mRNA and protein levels.

Kanemitsu et al. (2009c) further reported that Cdc25A protein, which is needed for the G1/S and S phase progression (Hoffmann et al., 1994; Jinno et al., 1994; Donzelli and Draetta, 2003), decreased at 36 and 48 hr. In a model of delayed S-phase progression, it is said that Chk1 and/or Chk2 proteins are phosphorylated (activated) in response to DNA damage, which leads to the disruption of Cdc25A protein, and thus inhibits the progression of S phase (Heffernan et al., 2002; Donzelli and Draetta, 2003). In addition, phosphorylated cdc2 protein and cyclin B1 protein increased from 24 to 48 hr, indicating the occurrence of G2/M arrest. Thus, 6-MP delays S-phase progression and induces G2/M arrest, resulting in apoptosis of NPCs in the fetal rat telencephalon. The accumulation of a G2/M-phase cell population may reflect the accumulation of G2-phase cell alone as indicated by the decrease in the number of phospho-histone H3-positive mitotic cells from 36 to 72 hr after treatment with 6-MP.

Kanemitsu et al. (2009c) also reported that the expression of puma and cleaved caspase-9 proteins, which are specific intrinsic pathway factors, increased in the rat telencephalon after 6-MP treatment, and that 6-MP-induced apoptosis of NPCs was completely absent in p53-deficient mice while the number of apoptotic NPCs was similar among Fas-mutated lpr/lpr and wild type mice. These findings suggest that the p53-mediated intrinsic pathway is essential for 6-MP-induced apoptosis of NPCs while the Fas pathway does not play a significant role in 6-MP-induced apoptosis of NPCs.

Etoposide (VP-16)

VP-16 is a semisynthetic derivative of podophyllotoxin, which is extracted from a plant Podophyllum peltatum and widely used as a chemotherapeutic agent for small cell lung cancer, testicular cancers and lymphomas.
VP-16 is also known to be a teratogen, and it brings on skeletal malformation and anomalies of the CNS in mice and rats when administered during the early gestational stage (Sieber et al., 1978). VP-16 interferes with topoisomerase II activity and causes DNA double-strand breaks through the formation of a DNA-drug-enzyme cleavage complex (Gantchev and Hunting, 1997; Hande, 1998).

In the study of Nam et al. (2010) on the neurotoxicity induced in the fetal mouse brain by transplacental exposure to VP-16 on GD12, the transition of the NPCs in the fetal brain was delayed as compared to that in the control, and some apoptotic cells were BrdU-positive, indicating that VP-16 damages proliferative NPCs, mainly S-phase cells. Flow-cytometric analysis showed that VP-16 induced S-phase accumulation and G2/M arrest at 4 and 8 hr, and then evoked apoptosis before NPCs enter the G2 and M phase, respectively. Differing from the ENU-induced S-phase accumulation of NPCs (Katayama et al., 2005a), the BrdU-incorporating potency of NPCs in the VP-16-exposed fetuses remained unchanged as compared to the control fetuses at 4 hr. Phosphorylation of ATM at Ser1981 and γH2AX detected by Western blot analysis indicates the occurrence of DNA damage following VP-16 treatment. p53 was also phosphorylated at Ser15 and 20 and increased after the activation of ATM kinase pathway. Western blot analysis also showed that VP-16 increased the levels of p53, p21, cyclin A, cyclin B1 and phospho-Cdc2 and decreased the Cdc25A level. It is supposed that Cdc25A degradation might induce the inhibition of S-phase progression and an increase in cyclin A might accelerate G1/S transition. In addition, it was considered that VP-induced G2/M arrest is caused by p21, which inactivates Cyclin B1-Cdc2 complex and eventually prevents mitotic entry (Fig. 7). Despite the p21-up-regulation, VP-16 caused only G2/M arrest, not G1 arrest, in the fetal brain.

The expression levels of p53-target genes (p21, fas and puma) mRNAs were elevated prior to the peak of apoptosis in NPCs (Nam et al., 2006b). As mentioned above, Puma is known to promote mitochondrial translocation and multimerization of Bax (Yu et al., 2003). However, the expression of bax mRNA was not increased despite the up-regulation of Puma mRNA by VP-16 treatment. In this context, it was reported that γ-irradiation-induced NPC apoptosis, both in vitro and in vivo, was critically dependent on p53 and caspase 9, but neither bax nor caspase 3 (D’Sa-Eipper et al., 2001). In addition, it was clarified that Noxa and Puma act as potentially key p53-dependent activators of the intrinsic apoptotic pathway in NPCs after genotoxic injury (Akhtar et al., 2006).

In the p53-deficient fetal mouse brain, G2/M arrest and apoptosis were almost abrogated, although S-phase accumulation still occurred. This suggests that VP-16-induced G2/M arrest and apoptosis are p53-dependent while VP-16-induced S-phase accumulation is p53-independent in the fetal mouse brain (Fig. 8). This also suggests that a decrease in Cdc25A after VP-16 treatment inhibits Cdk5 and finally blocks S-phase progression via the activation of an ATM-related and p53-independent pathway. In this context, it is reported that VP-16 has been shown to induce p21 up-regulation in a p53-dependent manner via ATM/ATR pathway in an in vitro experiment (Ding et al., 2003), and that cyclin A has been shown to be up-regulated in VP-16-treated tumor cells undergoing p53-dependent apoptosis (Rivera et al., 2006).

**REPAIR PROCESS FOLLOWING DAMAGE IN FETAL BRAIN**

In the adult brain, it is well known that the CNS harbors the ability to repair itself, and the activation of microglia and subsequent astrocytes are involved in the repair process (Fawcett and Asher, 1999; Silver and Miller, 2004). Further, there has been a recent focus on regenera-
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![Fig. 8](image)

Mechanisms of VP-16-induced neurotoxicity in the fetal mouse brain. VP-16 induces S-phase accumulation and G2/M arrest, resulting in apoptosis of NPCs. VP-16-induced G2/M arrest and apoptosis are p53-dependent but S-phase accumulation is p53-independent.

CP: cortical plate; VZ: ventricular zone.

Midkine (MK) is a heparin-binding growth factor that occurs as a product of a retinoic acid-inducible gene (Muramatsu, 1994). MK has been reported as involved in neurogenesis and neuron differentiation, in neurite outgrowth and neuronal survival (Michikawa et al., 1994). MK has been reported as involved in tissue repair (Muramatsu, 1994). MK seems to be essential for keeping the junctional complex of NPCs, and continuous MK expression in NPCs at the M phase may relate to the potential function of MK to maintain NPCs in a proliferative state to recover the lost population. Katayama et al. (2005b) also reported that the expression of two Id genes was elevated. Id proteins act as dominant negative antagonists of the basic helix-loop-helix (bHLH) family of the transcription factors, which positively regulate differentiation in many cell lineages (Ruzinova and Benezra, 2003), and bHLH factors are also known to have important roles in cell fate decisions during corticogenesis (Ross et al., 2003). Id proteins are expressed in NPCs in the developing CNS (Jen et al., 1997), and thought to maintain NPCs in an undifferentiated state by inhibiting bHLH factors (Ross et al., 2003; Iavarone and Lasorella, 2004). Id proteins work to maintain NPCs in a proliferative state to recover the lost population. Katayama et al. (2005b) also reported that the expression of osteopontin was prominently elevated in the repair phase. Osteopontin is expressed in macrophages of the developing brain and contributes to their migration and phagocytic function (Choi et al., 2004). Osteopontin plays an important role in the elimination of apoptotic cells by phagocytosis in the fetal brain.

Ueno et al. (2006c) offered important insights into the mechanisms of repair and regeneration in the developing brain after 5AzC-induced damage through histological and immunohistochemical examinations and DNA microarray analysis. Besides ENU, 5AzC also causes NPCs to synchronize in the S phase during recovery and then return to normal proliferation (Oyanagi et al., 1998; Ueno et al., 2006c), and this suggests that DNA repair might be coincident with S-phase retardation. The fetal brain maintains the capacity for repair and recovery of 5AzC-induced tissue damage (Ueno et al., 2006c), and damage seems to initiate regeneration more easily in the developing brain (Shimada and Langman, 1970; Houle and Das, 1984; Oyanagi et al., 1998; Ueno et al., 2006c) than in adults (Magavi et al., 2000; Doetsch, 2003; Picard-Riera et al., 2004). Moreover, after an injury, the plastic-
ity seen in fetal brains appears greater than that observed in adult brains. This is possibly due to greater proliferation of NPCs and the lack of astrocytic responses in the fetal brain. Astrocytes are generated in the late stage of the fetal period (Qian et al., 2000; Takizawa et al., 2001; San et al., 2003; Namihira et al., 2004), although astrocytes primarily mediate the repair of lesions in the adult brain with microglia (Fawcett and Asher, 1999; Silver and Miler, 2004).

Ueno et al. (2006c) reported that amoeboid microglia infiltrated the fetal rat brain and ingested the apoptotic cells during the repair period following 5AzC-treatment to dams on GD13. These microglial cells were positive for the multiple microglial markers such as ED-1 (Milligan et al., 1991), Iba-1 (Imai and Kohsaka, 2002), Lgals3/galecin3 (Walther et al., 2000; Sano et al., 2003) and osteoponin (Choi et al., 2004), which are said to be expressed in phagocytic cells. In addition, mRNAs for the microglia-related cytokines, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and macrophage colony stimulating factor (M-CSF), as well as Mif, a cytokine with proinflammatory effects (Calandra and Roger, 2003), were up-regulated. The respective genes play roles in the induction, proliferation and activation of microglia (Nakajima and Kohsaka, 2001; Hanisch, 2002), and their expression is also up-regulated by cyclophosphamide-induced injury to fetal brains (Hao et al., 2001a). TNF-α is expressed in microglia and NPCs, and CSF-1 receptor (CSF-1R), encoding an M-CSF receptor, in microglial cells (Hao et al., 2001a, 2001b and 2002). These reports indicate the importance of these cytokines for activating microglial cells in the developing brain. It is known that microglia release two types of opposing signaling molecules, cytokines and neurotropic factors (Nakajima and Kohsaka, 2001), so their effects on damaged tissue change depending on the local environment.

Up-regulated genes that are important for extracellular matrix, glycolysis and neural development were reported during the repair period following 5AzC-induced fetal brain damage (Ueno et al., 2006c). The extracellular matrix is important in the remodeling that occurs after tissue damage. Glycolysis is usually induced under hypoxic conditions, and chemical injury might induce hypoxia-like conditions, causing NPCs to up-regulate glycolytic genes. Hypoxia-inducible factors are the key transcription factors that respond to hypoxic conditions and control various target genes related to vascularization, glucose uptake/glycolysis, erythropoiesis, etc. (Bracken et al., 2003; Michiels, 2004). The expression of genes related to neural development is altered during the repair process, suggesting that tissue remodeling occurs (Ueno et al., 2006c). The normal, controlled expression patterns of these genes during brain development are critical for the formation of the complicated regional patterning of the brain (Rubenstein et al., 1998; Grove and Fukuchi-Shimogori, 2003).

Thus, the developing brain has the capacity to respond to the damage induced by extrinsic chemical stresses, by changing the expression of numerous genes and by recruiting microglia to aid the repair process (Fig. 9). The degree of damage induced by extrinsic stresses, and the extent of the subsequent repair process, would dramatically influence the level of abnormalities that would appear in the neonatal brain.

**CONCLUSIONS**

This paper reviewed the mechanisms of neurotoxicity induced in the developing brain of mice and rats by six chemicals which induce DNA damage in different ways. This paper also reviewed the repair process following damage in the developing brain.

The nuclei of proliferating NPCs undergo a characteristic migration in the VZ. Nuclear position within the VZ is correlated with cell cycle phase even when the cell cycle is disrupted, and the nuclei of NPCs can migrate only when their cell cycle is going. The location of apoptotic NPCs in the VZ and the time-course changes in the num-

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**Fig. 9.** Repair process following damage in the fetal brain. The developing brain has the capacity to respond to the damage by changing the expression of numerous genes related to glial cells, inflammation, extracellular matrix, glycolysis and neural development and by recruiting microglia to ingest apoptotic cells. Italic type: genes showing elevated expression.
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bers of apoptotic and mitotic NPCs are different among DNA-damaging chemicals, and such differences seem to reflect the differences in pharmacokinetics, mechanisms of DNA damage, signaling pathways involved in apoptosis and phases of cell cycle arrest among DNA-damaging chemicals.

ENU inhibits or arrests DNA replication in NPCs during the S phase. Both ENU-induced apoptosis and cell cycle perturbation in the S phase require p53, and ENU induces apoptosis in NPCs through the p53-mediated intrinsic pathway. HU directly inhibits DNA synthesis as a result of its inhibiting effect on ribonucleotide reductase. HU arrests NPC’s cell cycle first at the S checkpoint and then at the G2/M checkpoint, resulting in apoptosis of NPCs. HU-induced apoptosis may be mediated by p53 and caused through both intrinsic and extrinsic pathways. 5AzC forms a covalent bond with DNA methyltransferase when incorporated into DNA. 5AzC first induces the accumulation of cells in abnormal mitosis, G2-phase accumulation, and then apoptosis of NPCs. 5AzC-induced apoptosis is p53-dependent but another mechanism governs the G2/M checkpoint. At the G2/M checkpoint, abnormal activation of Cdc2 occurs following 5AzC-induced DNA damage and this may accelerate the G2 to M transition, probably resulting in accumulation of mitotic cells, some of which undergo mitotic catastrophe. Ara-C inhibits replicative DNA polymerases when incorporated into the end of growing DNA strand. Ara-C induces cell cycle arrest and apoptosis in NPCs at the S phase. The former is p53-independent while the latter is caused through p53-mediated intrinsic pathway. 6-MP is finally converted to thioguanine, which is incorporated into nucleic acid and induces DNA damage. 6-MP induces G2/M arrest, delays S-phase progression, and finally induces apoptosis in NPCs. The p53-mediated intrinsic pathway is essential for 6-MP-induced apoptosis while the Fas pathway does not play a significant role in 6-MP-induced apoptosis. VP-16 interferes with topoisomerase II activity and causes DNA double-strand breaks through the formation of a DNA-drug-enzyme cleavage complex. Following VP-16-induced DNA damage, ATM is first phosphorylated and then p53 is also phosphorylated through activation of the ATM kinase pathway. Subsequently, VP-16 induces S-phase accumulation and G2/M arrest, resulting in apoptosis of NPCs. VP-induced apoptosis and G2/M arrest are p53-dependent while S-phase accumulation is p53-independent.

As mentioned above, DNA-damaging chemicals reviewed in this paper induce NPC apoptosis without exception in a p53-mediated manner, but the cell cycle phase in which NPCs undergo apoptosis shows some differences. On the other hand, the phase and p53-dependency of cell cycle arrest are different among DNA-damaging chemicals. In addition, S-phase accumulation occurs after treatment with some DNA-damaging chemicals in p53-dependent or independent way. The developing brain has the capacity to respond to the damage induced by extrinsic chemical stresses, by changing the expression of numerous genes and by recruiting microglia to aid the repair process.

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