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Enhanced expression of cyclins and cyclin-dependent kinases in aniline-induced cell proliferation in rat spleen

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ABSTRACT

Aniline exposure is associated with toxicity to the spleen leading to splenomegaly, hyperplasia, fibrosis and a variety of sarcomas of the spleen on chronic exposure. In earlier studies, we have shown that aniline exposure leads to iron overload, oxidative stress and activation of redox-sensitive transcription factors, which could regulate various genes leading to a tumorigenic response in the spleen. However, molecular mechanisms leading to aniline-induced cellular proliferation in the spleen remain largely unknown. This study was, therefore, undertaken on the regulation of G1 phase cell cycle proteins (cyclins), expression of cyclin-dependent kinases (CDKs), phosphorylation of retinoblastoma protein (pRB) and cell proliferation in the spleen, in an experimental condition preceding a tumorigenic response. Male SD rats were treated with aniline (0.5 mmol/kg/day via drinking water) for 30 days (controls received drinking water only), and splenocyte proliferation, protein expression of G1 phase cyclins, CDKs and pRB were measured. Aniline treatment resulted in significant increases in splenocyte proliferation, based on cell counts, cell proliferation markers including proliferating cell nuclear antigen (PCNA), nuclear Ki67 protein (Ki67) and minichromosome maintenance (MCM), MTT assay and flow cytometric analysis. Western blot analysis of splenocyte proteins from aniline-treated rats showed significantly increased expression of cyclins D1, D2, D3 and E, as compared to the controls. Similarly, real-time PCR analysis showed significantly increased mRNA expression for cyclins D1, D2, D3 and E in the spleens of aniline-treated rats. The overexpression of these cyclins was associated with increases in the expression of CDK4, CDK6, CDK2 as well as phosphorylation of pRB protein. Our data suggest that increased expression of cyclins, CDKs and phosphorylation of pRB protein could be critical in cell proliferation, and may contribute to aniline-induced tumorigenic response in the spleen.

Introduction

Aniline, an aromatic amine, is a widely used industrial chemical with an annual production of over 1 billion pounds in the United States (Di Girolamo et al., 2009). Besides inducing hemolysis and hemolytic anemia, aniline exposure is also associated with damage to the spleen leading to splenomegaly, hyperplasia, fibrosis, and the eventual formation of highly malignant soft tissue or mesenchymal tumors, most commonly fibrosarcomas on chronic exposure in rats (Goodman et al., 1984; Weinberger et al., 1985; Bus and Popp, 1987; Khan et al., 1993, 1999a). Splenomegaly is one of the earliest characteristic features of aniline-induced splenotoxic responses preceding fibrosis and sarcomas (Weinberger et al., 1985; Bus and Popp, 1987; Khan et al., 1997, 1999a; Wang et al., 2005). As evidenced from previous studies, splenomegaly and splenotoxicity were associated with increased red pulp cellularity, increases in macrophages and fibroblasts, and such changes as iron overload, oxidative stress and activation of redox-sensitive transcription factors (Khan et al., 1997, 1999a,b, 2003a, 2006; Wang et al., 2005, 2008). However, molecular mechanisms leading to aniline-induced cellular proliferation in the spleen remain largely unknown.

Cell proliferation, a complex and tightly controlled process, plays a fundamental role in chemical-induced cell injury, including the injury which leads to neoplasia (Swenberg et al., 1983; Williams and Iatropoulos, 2002; Park et al., 2009). Increases in cell proliferation and changes in cell cycle are essential ingredients of many stages of chemical carcinogenesis (Emmendoerffer et al., 2000; Williams and Iatropoulos, 2002; Park et al., 2009). The phases of the cell cycle include G1 (Gap 1), S (synthesis of DNA), G2 (Gap 2) and M (mitosis). G0 is a phase of resting cells outside the cell cycle. When cells are stimulated by exogenous stimuli, they enter to the first gap phase (G1) from G0. Two of the most important group of proteins involved in the cell cycle machinery are cyclins and cyclin-dependent kinases (CDKs) (Murray, 2004; Chulu and Liu, 2009). Cell cycle progression is driven by changes in cyclin–CDK complexes, and if control of the cell cycle is disrupted, progress through the cycle might be stimulated by overexpressed cyclins, enhanced CDK activity or inactivated CDK inhibitors (Malumbres and Barbacid, 2001; Chulu and Liu, 2009). Induction of cyclins leads to their binding and

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activation of the associated CDK4, CDK6 or CDK2 (Hunter and Pines, 1994; Keyomarsi et al., 1995; Sherr, 1995; Malumbres and Barbacid, 2001). In mammalian cells, cyclin D–CDK4/6 and cyclin E–CDK2 complexes are required to promote cell cycle entrance from quiescence, progression through the G1 phase and transition from G1 into S phase in response to mitogenic stimulation (Pardee, 1989; Malumbres and Barbacid, 2001; Chulu and Liu, 2009). Deregulation of G1 cyclins is associated with tumorigenesis, and altered CDKs in G1 phase can also contribute to oncogenic process (Hunter and Pines, 1994; Keyomarsi et al., 1995; Malumbres and Barbacid, 2009).

The G1 phase of the cell cycle is a functional period during which cells prepare for S phase, and the control of post-embryonic cell proliferation occurs before S phase (Pardee, 1989; Lundberg and Weinberg, 1999). There is a checkpoint in the G1-S transition, termed as 'restriction point' or 'R point' (Pardee, 1989), and R point is a central event in normal cellular proliferation control (Lundberg and Weinberg, 1999), because it determines if a normal proliferating cell in G1 will continue to cycle or will revert to quiescence. The retinoblastoma proteins (pRB) are best known for their roles in restraining the G1-S transition through the regulation of E2 transcription factor (E2F)-responsive genes. There are a variety of cyclin/CDK complexes formed during distinct phases and time windows of the cell cycle; however, they may both get co-overexpressed and modify pRB (Weinberg, 1995; Lundberg and Weinberg, 1998, 1999). Cyclin D–CDK4/6 and cyclin E–CDK2 complexes sequentially phosphorylate the pRB (Hatakeyama et al., 1994; Lundberg and Weinberg, 1998; Harbour et al., 1999), and the hyper-phosphorylated proteins release the E2F transcription factors that are required for the S-phase entry (Sherr and Roberts, 1999; Sherr, 2000).

The precise molecular mechanisms in aniline-induced splenotoxicity, especially the molecular events in splenocyte proliferation and expression of the proteins which regulate cycling cells, are unknown. This study was, therefore, focused on evaluating the cellular proliferation and expression of cell cycle proteins, especially G1 phase cyclins and CDKs in an experimental condition preceding a tumorigenic response, but known to induce iron overload and oxidative stress in the spleen (Khan et al., 1999a, 1999b; Wang et al., 2008) following aniline insult.

Materials and methods

Animals and treatments

Male Sprague-Dawley rats (~200 g), obtained from Harlan Sprague-Dawley (Indianapolis, IN), were housed in wire-bottom cages over adsorbent paper with free access to tap water and Purina lab chow and maintained in a controlled environment animal room (temperature, 22 °C; relative humidity, 50%; photoperiod, 12-h light/dark cycle) for 7 days prior to the treatments. The experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at University of Texas Medical Branch. The animals were divided into two groups of six each. One group of animals received 0.5 mmol/kg/day aniline hydrochloride (~97%; Aldrich, Milwaukee, WI) via drinking water (pH of the solution adjusted to ~6.8), while the other group received water only and served as controls (Khan et al., 1993, 1999a,b, 2003a, 2006; Wang et al., 2005; Ma et al., 2008). The drinking pattern of experimental and control rats was similar. Choice of dose and duration of exposure was based on earlier studies (Khan et al., 1993, 1999a,b, 2006; Wang et al., 2005; Ma et al., 2008). After 30 days, the animals were euthanized under nembutal (sodium pentobarbital) anesthesia and the spleens were aseptically removed, weighed and divided into several portions for use in various analyses. Portions of the spleen were snap-frozen in liquid nitrogen and stored at −80 °C for RNA isolation and protein extraction, whereas another portion of the spleen was processed to obtain splenocytes.

Isolation and culture of splenocytes

A portion of the spleen was passed through a cell strainer (BD Biosciences, Bedford, MA) in RPMI 1640 culture medium. The cell suspension was centrifuged at 1000*g* for 5 min at 4 °C. The cells were resuspended with Hanks' balanced salt solution (HBSS) without calcium and magnesium. The splenocytes were isolated as described earlier (Khan et al., 2006; Wang et al., 2008). Briefly, the suspended cells were layered onto 6 ml of Histopaque 1083 (Sigma Chemical Co., St. Louis, MO) and centrifuged at 700*g* for 30 min at 20 °C. After centrifugation, the splenocyte layers were carefully removed, transferred to 50 ml Falcon plastic tubes, and washed twice with 20 ml HBSS. The cells were resuspended in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin and 10% heat inactivated FBS (Sigma). Splenocytes were counted microscopically and the cell viability was determined by trypan blue exclusion method. The viability of splenocytes isolated from both control and aniline-treated rats was more than 95%.

MTT assay

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was dissolved in phosphate buffered saline (PBS, 5 mg/ml) and filtered through 0.22 μm filter. The MTT assay was done as described earlier (Mosmann, 1983) with minor modifications. Briefly, the isolated splenocytes, suspended in complete RPMI 1640, were plated in 96-well flat-bottom plates (1×10^5/100 μl/well) (Corning Inc., Corning, NY) and cultured in a humidified incubator with 5% CO_2 at 37 °C for 24, 48 and 72 h. After addition of 20 μl MTT solution in each well, the incubation was continued for another 4 h at 37 °C. One hundred microliters of stop solution (10% SDS-0.01 N HCl) was then added to each well, and the absorbance was read at 570 nm (test) and 630 nm (reference) 5 min later.

Flow cytometric analysis for splenocyte proliferation

Splenocytes isolated from control and aniline-treated rats were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using the CellTrace CFSE Cell Proliferation kit (Molecular Probes, Eugene, OR) and then plated into 24-well flat-bottom plates at 1×10^6/well in a total volume of 1 ml. The splenocytes were incubated at 37 °C with 5% CO_2, and after 72 h, harvested for flow cytometric analysis using a Becton-Dickinson FacsCanto flow cytometer (BD Biosciences, San Jose, CA).

Preparation of total protein extracts

Total spleen tissue lysates were prepared by using the lysis buffer essentially as described by the manufacturer (Cell Signaling, Beverly, MA). Protein concentration in the lysates was determined by Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). The lysates were used for the detection of cyclin D1, cyclin D2, cyclin D3, cyclin E, CDK2, CDK4, CDK6, pRB and phospho-pRB proteins.

Preparation of nuclear protein extracts (NEs)

NEs were prepared essentially as described earlier (Ma et al., 2008). Spleen tissues (from control and aniline-treated rats) were cut into small pieces, homogenized briefly with a loose glass pestle in cold hypotonic buffer [10 mM HEPES-KOH, 10 mM KCl, 100 μM EDTA, 100 μM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml pepstatin, and a complete protease inhibitor cocktail (Roche, Germany)], and incubated on ice for 20 min. Tissues were then further homogenized using a tight pestle and centrifuged at 800*g* for 4 min to obtain nuclear pellets. Pellets were gently washed two times with homogenizing buffer. Nuclear proteins were extracted in a high salt buffer (20 mM HEPES–