Communication

Inhibition of Nuclear Factor-κB Activity Is Involved in E1A-mediated Sensitization of Radiation-induced Apoptosis*

(Received for publication, August 5, 1997, and in revised form, October 5, 1997)

Ruping Shao‡‡, Devarajan Karunagaran‡‡‡, Binhua P. Zhou‡, Kaiyi Li, Su-Shun Lo††, Jiong Deng§, Paul Chiao, and Mien-Chie Hung†††‡‡‡

From the ‡Department of Tumor Biology, Breast Cancer Research Program and †Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

The adenoviral E1A protein has been implicated in the potentiation of apoptosis induced by various external stimuli, but the exact mechanism of that potentiation is not clear. In this study, we compared the sensitivity to ionizing γ-irradiation of E1A transfectants with that of parental cells in a human ovarian cancer cell line (SKOV3.ip1); we found that the E1A transfectants became sensitive to radiation-induced apoptosis. Recently, activation of the transcription factor nuclear factor-κB (NF-κB) has been shown to play a key role in the anti-apoptotic pathway of radiation-induced apoptosis. In an attempt to determine whether NF-κB was involved in the E1A-mediated sensitization of radiation-induced apoptosis, we found that radiation-induced activation of NF-κB occurred in the parental cells but was blocked in the E1A transfectants. Furthermore, parental cells co-transfected with NF-κB and E1A were better protected from undergoing apoptosis upon irradiation than those transfected with E1A alone. Thus, our results suggest that inhibition of NF-κB activation by E1A is a plausible mechanism for E1A-mediated sensitization of radiation-induced apoptosis.

In the past, E1A was considered to be an oncogene, because it immortalizes primary rodent cells and transforms them in cooperation with a second oncogene such as E1B or activated ras (1), but no evidence has been found to suggest that an oncogenic role exists for E1A in human cancers. Recent findings indicate that E1A can function as an anti-oncogene to suppress transformation, metastasis, and tumorigenicity as demonstrated previously (2–6). E1A also has been reported to increase cellular susceptibility to apoptosis, particularly under the conditions of serum starvation or high cell density (7). Furthermore, E1A-expressing derivatives of primary mouse embryo fibroblasts were shown to undergo rapid apoptosis following treatment with ionizing radiation or with several chemotherapeutic compounds (8). Apoptosis is an active physiological process that can be triggered by a wide variety of cellular stresses, including DNA damage, ultraviolet (UV) radiation, ionizing radiation, heat shock, and oxidative stress, as well as by extracellular stimuli acting through the cell-surface receptors (9). The mechanisms by which E1A sensitizes cells that undergo apoptosis in response to various stimuli are not clear.

Many apoptotic stimuli, including tumor necrosis factor-α, ionizing radiation, and several chemotherapeutic compounds, activate NF-κB, a pleiotropic transcription factor that regulates many genes, such as immunoreceptors, cytokines, viruses, and others (10). Recently, NF-κB has been shown to have an anti-apoptotic role, blocking apoptosis induced by tumor necrosis factor-α, ionizing radiation, or the chemotherapeutic agent daunorubicin (11–14).

In light of our observation and reported findings, we hypothesized that NF-κB may play a protective role in the E1A-mediated sensitization of cells to apoptotic stimuli. Thus, we tested E1A transfectants of human cancer cell lines for their sensitivity to ionizing γ-irradiation. The cells transfected with E1A were highly sensitive to radiation-induced apoptosis based on in vitro growth studies, DNA fragmentation assays, and fluorescence-activated cell sorter analysis. In addition, we found that NF-κB inactivation is required in the E1A-mediated sensitization of radiation-induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Cultures—The SKOV3.ip1 cell line was generated from ascites developed in nu/nu mouse by administering an intraperitoneal injection of SK-OV-3, a human ovarian carcinoma cell line obtained from the American Type Culture Collection (6). Cells were grown in Dulbecco’s modified Eagle’s medium/F-12 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. The p1-E1A2 and p1-E1A-Eis cells were established by transfecting the SKOV3.ip1 cells with E1A-expressing pE1A plasmid DNA and pE1A-dl343 containing a 2-base pair frameshift deletion in the E1A coding region, respectively. The transfecteds were grown under the same conditions, except that G418 (800 μg/ml) was added to the culture medium. Cells were irradiated with a 137Cs source emitting a fixed dose rate of the indicated Gy (Irradiator model 0103, U. S. Nuclear Corp.).

In Vitro Growth Rate Analysis—The in vitro growth rates of the cell lines were assessed by counting the cells using a Coulter counter or by MTT assay (15). For MTT assays, cells (3 × 104/well) were plated in 96-well culture plates in 0.2 ml of culture medium and allowed to adhere for 2 h; 20 μl of MTT was then added to each well. Cells were cultured for an additional 2 h, and 100 μl of lysis buffer (20% sodium dodecyl sulfate (SDS) in 50% N,N-dimethylformamide, pH 4.7) was added. The cells were incubated overnight, and absorbance at 570 nm was measured. For cell counting, samples of 4 × 105 cells with or without γ-radiation were plated in triplicate in a six-well plate. After different time intervals, the cells were harvested and counted using a Coulter counter.

Immunoblotting—Immunoblot analysis was performed as described previously (6). A monoclonal antibody against the E1A proteins, M58 (PharMingen), was used.

* This work was supported by Grants R01-CA58880 and R01-CA60658 (to M.-C. H.) from the National Institutes of Health, Core Grant 16672 from the National Cancer Institute, and by the Nellie Connally Breast Cancer Research Fund at M. D. Anderson Cancer Center (to M.-C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The first two authors contributed equally to this work.

§ Visiting scientist financially supported in part by the Veterans General Hospital, Taipei, Taiwan.

¶ To whom correspondence should be addressed: Dept. of Tumor Biology, Box 79, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-792-3668; Fax: 713-794-4784.

1 The abbreviations used are: NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RelA, a p65 subunit of NF-κB; Gy, gray(s).
in a 35-mm well were transfected with 2.2 μg of plasmid DNA containing NF-κB binding sites from human immunodeficiency virus (20). Binding of the mutant NF-κB competitor was a double-stranded oligonucleotide containing the NF-κB site from human immunodeficiency virus (20). 1.5 μg of total DNA (RelA or pukE1A, 0.4 μg) was added to each well. All the experiments were performed using an MTT assay. We used a human ovarian cancer cell line derivative, SKOV3.ip1, that was stably transfected with either wild-type Ad5 E1A (ip1-E1A2) or an E1A frameshift Efs mutant (ip1-Efs) (6). When the cells were irradiated, the E1A transfectants showed a more significant decrease in growth rate than the parental or Efs transfectants did when they were irradiated under the same conditions during a 3-day period (Fig. 1A). An optimal radiation dose of 5 Gy was chosen after the cells were irradiated with varying doses (data not shown). In the absence of radiation, the growth rates of the parental and Efs transfectants were similar, and the growth rate of the E1A transfectant was slightly lower (6). The changes in DNA replication rate.

RESULTS AND DISCUSSION

To test whether E1A can sensitize cells to radiation-induced changes during growth, in vitro growth rate analysis was first performed using an MTT assay. We used a human ovarian cancer cell line derivative, SKOV3.ip1, that was stably transfected with either wild-type Ad5 E1A (ip1-E1A2) or an E1A frameshift Efs mutant (ip1-Efs) (6). When the cells were irradiated, the E1A transfectants showed a more significant decrease in growth rate than the parental or Efs transfectants did when they were irradiated under the same conditions during a 3-day period (Fig. 1A). An optimal radiation dose of 5 Gy was chosen after the cells were irradiated with varying doses (data not shown). In the absence of radiation, the growth rates of the parental and Efs transfectants were similar, and the growth rate of the E1A transfectant was slightly lower (6). The changes...
in growth upon radiation were confirmed in another assay by counting the cells before and after irradiation during the same period of time (Fig. 1B). As a control, we also checked the expression of E1A in these cell lines by immunoblot analysis using a monoclonal antibody (M58); the results showed that the ip1-E1A2 cells expressed E1A but the parental (SKOV3.ip1) and mutant (ip1-Efs) cells did not (inset, Fig. 1). These results suggest that the introduction of E1A into SKOV3.ip1 cells significantly facilitates the radiation-induced retardation of cell growth. However, measurement of DNA synthesis rates using the [3H]thymidine incorporation assay revealed no significant difference between the parental and E1A-transfected cells after irradiation when the radioactive counts were normalized against the counts in the nonirradiated control (Fig. 1C). Presumably, the net cell-growth rate depends on a fine balance between the cell-replication rate and the cell-death rate. The fact that cell replication requires DNA synthesis and that the effect of radiation on DNA synthesis does not change significantly between E1A transfectants and parental cells (Fig. 1C) indicates that the effect of radiation on cell replication cannot account for the differential response to radiation between E1A transfectants and parental cells (Fig. 1, A and B).

Therefore, a change in cell death is most likely to be the major cause of the differential response to radiation during cell growth between the E1A transfectants and the parental cells (Fig. 1, A and B). To study the apoptotic changes, cells were irradiated and analyzed by DNA fragmentation analysis. Indeed, the E1A-transfected cells showed dramatic changes in DNA fragmentation after irradiation for 24 h, but the parental cells (SKOV3.ip1) did not exhibit DNA fragmentation even after irradiation for 48 h at doses of 5 or 10 Gy (Fig. 2A), which is reminiscent of the often-seen resistance to radiotherapy among cancer patients. The 180–200-base pair DNA fragments

FIG. 2. Ionizing radiation induces DNA fragmentation and apoptosis in ip1-E1A2 cells but not in SKOV3.ip1 cells. A, cells were irradiated (0, 5, or 10 Gy) and cultured for the indicated period of time and the DNA was extracted and analyzed by gel electrophoresis. B, SKOV3.ip1 and ip1-E1A2 cells were irradiated at a dose of 5 Gy, and quantitation of apoptotic cells, expressed as a percentage, was done at the time intervals shown using flow cytometric analysis of the sub-G1 phase. Symbols: □, SKOV3.ip1; ■, ip1-E1A2.
and the multiples of them that were observed in this study represent a characteristic fragmentation pattern attributed to apoptotic phenomena (21, 22). To quantitate the apoptotic changes observed in E1A-transfected cells upon irradiation, we measured the sub-G1 population of cells by flow cytometry. As shown in Fig. 2B, more than 30% of the E1A-transfected cells showed the apoptotic peak observed at 72 h after irradiation, while the apoptotic population in the parental cells was less than 5% during the same period. A similar but more profound trend was also observed with the administration of a higher dose of 10 Gy (data not shown). Thus, our results are consistent with the notion that E1A can sensitize cells to radiation-induced apoptotic changes.

Ionizing radiation is known to induce the activation of RelA (p65), a subunit of NF-κB in many systems (14, 23, 24), but the involvement of ionizing radiation in the E1A-mediated susceptibility of cells to radiation has not been examined. Therefore, we analyzed SKOV3.ip1 cells and their E1A transfectants for RelA DNA binding activity before and after irradiation. The results indicate that ionizing radiation induces NF-κB DNA binding activity in parental cells but not in E1A-transfected cells (Fig. 3A). Increasing the radiation dose to 10 Gy did not activate RelA in the E1A-transfected cells, and there was no further increase in activation in the parental cells (data not shown). The activated complex induced by radiation in the SKOV3.ip1 cells was abolished in the presence of a wild-type oligonucleotide, but a mutant oligonucleotide failed to displace the active complex, indicating the specificity of the DNA binding activity (Fig. 3B). As reported previously (13, 25), the two specific DNA-protein complexes probably represent the RelA and p50 heterodimers and the p50 homodimer, respectively. To specifically DNA-protein complexes probably represent the RelA and p50 heterodimers, and the p50 homodimer, respectively. To test this possibility, we transiently transfected the E1A and lacZ genes into SKOV3.ip1 cells both with and without the RelA expression plasmid, irradiated the cells, and analyzed the surviving cells by staining the cells with 5-bromo-4-chloro-3-indol-β-D-galactoside. This approach, which uses the number of β-galactosidase staining cells as an index of the surviving cells, has been used routinely by others to demonstrate the protective role of RelA against stress-induced apoptosis. The results show that RelA indeed can prevent at least part of the E1A transfectants from undergoing apoptosis upon irradiation (Fig. 4). Taken together, our results strongly suggest that RelA plays a role in protecting cells from ionizing radiation-induced apoptosis, and the E1A-mediated inactivation of RelA contributes significantly to the E1A-mediated sensitization of radiation-induced apoptosis.

REFERENCES
1. Ruley, H. E. (1990) Cancer Cells 2, 258–268
2. Chang, J. Y., Xia, W., Shao, R., and Hung, M. C. (1996) Oncogene 13, 1405–1412
3. Chen, H., Yu, D. H., Chinnadurai, G., Karunagaran, D., and Hung, M. C. (1997) Oncogene 14, 1965–1971
4. Chinnadurai, G. (1992) Oncogene 7, 1255–1258
5. Frisch, S. M. (1996) Mutat. Res. 350, 261–266
6. Yu, D., Wolf, J. R., Scandola, M., Price, J. E., and Hung, M. C. (1993) Cancer Res. 53, 891–898
7. Rao, L., Debbas, M., Sabbatini, P., Hegenberg, D., Korsmeyer, S., and White, E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7742–7746
8. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993) Cell 74, 957–967
9. Coulie, S., and Clarke, P. (1996) Curr. Biol. 6, 1586–1588
10. Baichwal, V. R., and Basu, P. A. (1997) Curr. Biol. 7, R94–R96
11. Beg, A. A., and Baltimore, D. (1996) Science 274, 782–784
12. Liu, Z. G., Hau, H., Goeddel, D. V., and Karin, M. (1996) Cell 87, 555–576
13. Van Antwerp, D. J., Martin, S. J., Kadri, T., Green, D. R., and Verma, I. M. (1996) Science 274, 787–789
14. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) Science 274, 784–787
15. Alley, M. C., Scrudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R. H., and Boyd, M. R. (1998) Cancer Res. 48, 589–601
16. White, E., Grodzicker, T., and Stillman, B. W. (1984) J. Virol. 52, 410–419
17. Bouras, V., Burt, F. R., Brown, K., Villabobo, J., Park, S., Hnneck, P. R., Bravo, R., Kelly, K., and Siebenlist, U. (1992) Mol. Cell. Biol. 12, 685–695
18. Chang, J. Y., Xia, W., Shao, R., Sorgi, F., Hertogebagyi, G. N., Huang, L., and Hung, M. C. (1997) Oncogene 14, 561–568
19. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 2499
20. Cauley, K., and Verma, I. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 390–394
21. Sanchez-Prieto, R., Quintanilla, M., Anaya, A., and Ramon y Cajal, S. (1996) Oncogene 15, 1083–1092
22. Bracey, T. S., Miller, J. C., Preece, A., and Paraskeva, C. (1995) Oncogene 10, 2391–2396
23. Brach, M. A., Hass, R., Sherman, M. L., Gunji, H., Weichelbaum, R., and Kufe, D. (1991) J. Clin. Invest. 88, 691–695
24. Singh, S. P., and Lavin, M. F. (1990) Mol. Cell. Biol. 10, 5279–5285
25. Cadoret, A., Bertrand, F., Baron-Delage, S., Levy, P., Courtois, G., Gespach, C., Capeau, J., and Cherqui, G. (1997) Oncogene 14, 1588–1600