Abnormal Expression of the p53-Binding Protein MDM2 in Hodgkin’s Disease

By Marco Chilosi, Claudio Doglioni, Fabio Menestrina, Licia Montagna, Antonella Rigo, Maurizio Lestani, Mattia Barbareschi, Aldo Scarpa, Gian Mario Mariuuzzi, and Giovanni Pizzolo

The possible involvement of p53 tumor suppressor gene in the pathogenesis of Hodgkin’s disease (HD) is suggested by the frequent finding of abnormal accumulation of p53 protein in the nuclei of Reed-Sternberg cells and their variants (H-RS) in a large proportion of cases. This finding, besides being consistent with the presence of p53 gene mutations, might represent a consequence of the inactivating interaction between p53 and p53-binding proteins such as the product of the MDM2 cellular oncogene. We have examined an unsel ected series of 77 HD cases of different histologic patterns for the expression of p53 and MDM2 proteins, using specific monoclonal antibodies and sensitive immunohistochemical techniques in single- and double-marker combinations. In the large majority of cases (66/77), a variable proportion of H-RS cells expressed MDM2 that was strictly confined to the nuclei. Coexpression of both MDM2 and p53 was common in the same cells. The abnormal nuclear expression of p53 and MDM2 did not seem to correlate with the presence of Epstein-Barr virus infection, as shown by the results of LMP-1 antigen expression and EBER in situ hybridization analysis. Our data suggest that the abnormal accumulation of MDM2 and p53 proteins in HD might reflect a derangement of molecular mechanisms that could play a pathogenetic role in this disease.

© 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Samples composition. Seventy-seven cases of histologically proven HD collected from the files of the Department of Pathology of Verona University were studied. Cases were classified according to the Rye classification on the basis of morphologic and immunophenotypic features. Accordingly, 63 cases were diagnosed as HD of the nodular sclerosis type (NS), 10 cases as HD of the mixed cellularity type (MC), and 4 cases as nodular lymphocyte predominant (NLP). Sixty-seven samples were from lymph nodes, 9 from mediastinal masses, and 1 from a liver biopsy.

From the Istituto di Anatomia Patologica and Cattedra di Ematologia, Università di Verona, Verona; and Anatomia Patologica Ospedale Civile di Trento and Feltre, Feltre, Italy.


Supported by grants from AIRC (Milan) and the Italian National Research Council, Special Project “Clinical Application of Oncological Research.”

Address reprint requests to Marco Chilosi, DCh, MD, Istituto di Anatomia Patologica, Università di Verona, Policlinico Borgo-Roma, 37134 Verona, Italy.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.

0006-4971/94/8412-0036$3.00/0

Blood, Vol 84, No 12 (December 15), 1994; pp 4295-4300
Sample immunostaining. On the basis of the results obtained in preliminary experiments, the immunohistochemical analysis for p53 and MDM2 was performed on formalin-fixed (<24 hours of fixation) paraffin-embedded material. Briefly, sections were deparaffinized in xylene, rehydrated, washed in phosphate-buffered saline (PBS), immersed in 0.01 mol/L citrate buffer, pH 6, and irradiated in a microwave oven for 5 minutes at 750 W (3 times) and for 10 minutes at 600 W (1 time). The sections were then kept for 15 minutes at room temperature before further PBS washing and immunostaining. Monoclonal antibodies (MoAbs) DO-1 (Dako, Glostrup, Denmark) and IF-2 (Oncogene Science, Uniondale, NY) were used for detecting p53 and MDM2, respectively. A similar immunohistochemical procedure was used for investigating CD30 (MoAb Ber-H2; Dako) and the EBV-encoded LMP-1 antigen (MoAb CS1-4; Dako). Double-marker analyses with p53/CD30, MDM2/CD30, and p53/MDM2 MoAbs were performed on paraffin sections using previously detailed techniques. After incubation, one antibody was shown using the avidin-biotin-peroxidase system (LSAB: Dako), developed with H2O2 and 3,3′-diaminobenzidine, or the alkaline phosphatase antialkaline phosphatase (APAAP) technique, using the NBT/BCIP cytochemical staining (ie. 10 mg nitroblue tetrazolium dissolved in 200 μL N.N-dimethylformamide [Sigma, St Louis, MO] and 5 mg 5-bromo-4-chloro-3-indolyl phosphate dissolved in 200 μL N,N-dimethylformamide [Sigma]) in 31 mL of 0.1 mol/L, pH 9.5, Tris-buffer solution containing 0.1 mol/L NaCl, 0.005 mol/L MgCl2. The reactivity with the second MoAb was shown by the AAPAP technique using the cytochemical staining with Naphtol AS Bi Phosphate substrate and either exaharized new-fuscin or Fast Blue BB salt as chromogens to obtain a clear-cut chromatric contrast between different stainings. A semiquantitative evaluation of cells expressing the two antigens was performed using double-marker preparations, giving different scores on the basis of the percentage of positive cells within the morphologically recognizable H-RS cells: –, absent; –, rare (<10%); ++, scattered (10% to 20%); ++, numerous (>20% to >50%); and ++++, most (>50% to 100%).

Controls. Reactive lymph nodes (10 samples), normal infant thymus (2 samples), and bone marrow biopsies obtained in the context of staging procedures (10 samples) were used as controls.

EBV in situ hybridization. The presence of small nonpolyadenylated RNA particles were detected using an in situ hybridization technique. EBER1- and EBER2-specific fragments were provided by Dr A.B. Rickinson (Birmingham, UK). Digoxigenin-labeled antisense or sense (control) run-off transcripts were generated using either T3 or T7 polymerases, respectively, and digoxigenin-11-uridine-5′-triphosphate (Boehringer Mannheim, Mannheim, Germany). A mixture of EBER1- and EBER2-specific RNA probes was applied to increase the sensitivity. Paraffin-embedded sections were deparaffinized in xylene and ethanol and washed in PBS. Proteolysis was performed at room temperature through 10 minutes of incubation with 1 mg/mL of proteinase K (Boehringer Mannheim) followed by two washes in PBS and postfixation in 4% paraformaldehyde for 10 minutes. Sections were then dehydrated in ethanol. Sections were covered with 50 μL of hybridization mixture (50% deionized formamide, 0.1× Denhardt’s solution, and 20% dextran sulfate) containing 5 to 10 ng of probe and incubated overnight at 37°C under coverslips. After detaching coverslips, sections were washed in TBS/0.1% Triton X-100 (twice for 10 minutes) and treated with 20 μg/mL of RNaseA (10 minutes) to digest non-specifically bound probe. Immobilized digoxigenin was detected using a monoclonal digoxigenin-specific antibody (Boehringer Mannheim) diluted 1:50 and the APAAP procedure. The specificity of the EBER probes was tested by abolition of hybridization after pretreatment of sections with RNaseA.

RESULTS

In control samples, nuclear immunostaining for MDM2 and p53 was only detected in rare lymphoid cells within germinal centers of reactive lymph nodes, in cortical areas of infant thymus samples, and in scattered erythroblasts in bone marrow samples. All control samples were negative for LMP-1 immunostaining and EBER in situ hybridization.

The results of immunohistochemical analysis of HD issues for MDM2 and p53 are summarized in Table 1. In the large majority of cases (66/77), a variable proportion of cells, recognized as H-RS on the basis of morphology and membrane/cytoplasmic immunoreactivity for CD30 or CD15, showed MDM2 immunoreactivity (Fig 1). p53 was expressed in 61 of 77 cases (Table 1 and Figs 2 and 3). The immunostaining for both MDM2 and p53 was of variable intensity in different cells and in different samples, but was strictly confined to the nuclei of H-RS cells. Frequently, multinucleated RS cells exhibited asymmetric expression of MDM2 and p53, with immunoreactivity confined to one nucleus. In some H-RS, immunoreactivity was observed in small round nuclear fragments (Fig 3). The analysis of MDM2 and p53 reactivity pattern showed a similar proportion of cells expressing the two molecules in 27 of 77 cases (immunoreactivity type I), excess of p53-positive cells in 19 cases (type II), excess of MDM2 expression in 23 cases (type III), and 8 cases negative for both antigens (type IV; Table 1). A relevant proportion (30% to 80%) of H-RS cells coexpressing both MDM2 and p53 were present in cases of types I through III, as shown on double-staining preparations. Coexpression of both MDM2 and p53 in H-RS was common in mononuclear Hodgkin’s cells, whereas an asymmetrical immunoreactivity was common in multinucleated H-RS cells (Figs 4 and 5). The abnormal expression of both p53 and MDM2 was observed in the majority of cases of the nodular sclerosis (48/63) and mixed cellularity (9/10) types, but was less common in lymphocyte predominance type (1/4). The expression of EBV-related antigen (LMP-1) and/or RNA (EBER-1) was shown in a minority of cases (12/72), all but 1 characterized by the presence of MDM2 positive H-RS. A proportion of these EBV+ cases (8/12) contained also p53+ H-RS. The one EBV+/MDM2− case (no. 7, Table 1) was secondary to immunodeficiency because of human immunodeficiency virus (HIV) infection.

DISCUSSION

In this study, we showed an abnormal accumulation of the MDM2-encoded protein in a variable proportion of neoplastic H-RS cells in the majority of cases of HD (66/77). MDM2 immunoreactivity was restricted to the nuclei of H-RS. Double-staining techniques showed that MDM2 was often overexpressed together with p53 protein in the same cells.

The first practical information derived from our study is that the immunohistochemical approach to the analysis of MDM2 expression is highly informative in a condition such as HD in which the neoplastic H-RS cells are sparse and molecular biology studies are cumbersome and scarcely reproducible. More interestingly, our findings raise the point
of the biologic meaning of MDM2 protein expression in relationship with p53 accumulation in neoplastic cells and, in particular, in HD.

Recent studies have shown that the MDM2 gene is a target for activation by wild-type p53 and that an autoregulatory feedback loop exists that regulates both the activity of the p53 protein and the expression of the MDM2 gene.26-27 The interaction between p53 and MDM2 in normal and neoplastic cells seems quite complex because different polyopeptides may arise from different spliced mRNA forms of the MDM2 gene, posttranslational modifications of the MDM2 protein, or the use of alternative promoters.28 Further complexity is added by the different timing of expression of p53 and MDM2 after DNA damage.26 The heterogenous pattern of immunostaining for p53 and MDM2 observed in different cases and within the same samples and the observation of cases with few positive cells or not showing the abnormal expression of p53 and MDM2 could be either related to this complexity or to intratumor heterogeneity. In addition, alterations of the half-lives of p53 and MDM2 reaching in variable proportions the threshold of sensitivity of the technique13 could interfere with their immunohistochemical evidence at the nuclear level.

Clearly, the biologic significance of the abnormal expression of p53 and MDM2 in HD can only be matter of speculation. The emergence of neoplastic H-RS, at least in a propor-

### Table 1. Expression of p53, MDM2, and EBV in Hodgkin’s and Reed-Sternberg Cells in 77 Cases of HD of Different Types, Ordered After MDM2 Immunoreactivity

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yr)</th>
<th>Type</th>
<th>Tissue</th>
<th>p53</th>
<th>Mdm2</th>
<th>EBV</th>
<th>Case No.</th>
<th>Age (yr)</th>
<th>Type</th>
<th>Tissue</th>
<th>p53</th>
<th>Mdm2</th>
<th>EBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B.M.</td>
<td>22</td>
<td>NS</td>
<td>LN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2. B.G.</td>
<td>27</td>
<td>NS</td>
<td>LN</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2. C.M.</td>
<td>42</td>
<td>NS</td>
<td>LN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3. T.G.F.</td>
<td>70</td>
<td>NS</td>
<td>LN</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3. F.A.</td>
<td>56</td>
<td>NS</td>
<td>LN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4. B.C.</td>
<td>93</td>
<td>NS</td>
<td>LN</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4. F.E.</td>
<td>19</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>5. T.E.</td>
<td>36</td>
<td>NS</td>
<td>LN</td>
<td>+++</td>
<td>-</td>
<td>(HIV+)</td>
</tr>
<tr>
<td>6. D.L.</td>
<td>23</td>
<td>NS</td>
<td>LN</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>7. D.L.</td>
<td>38</td>
<td>NS</td>
<td>LN</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8. F.D.</td>
<td>27</td>
<td>NS</td>
<td>LN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9. Z.G.</td>
<td>52</td>
<td>NS</td>
<td>MED</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10. Z.G.</td>
<td>52</td>
<td>NS</td>
<td>MED</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11. R.K.</td>
<td>15</td>
<td>NS</td>
<td>LN</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12. S.L.</td>
<td>19</td>
<td>NS</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>13. M.L.</td>
<td>19</td>
<td>NS</td>
<td>MED</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14. S.L.</td>
<td>19</td>
<td>NS</td>
<td>MED</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>15. V.S.</td>
<td>24</td>
<td>NS</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16. Z.C.</td>
<td>26</td>
<td>NS</td>
<td>LN</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>17. Z.L.</td>
<td>27</td>
<td>NS</td>
<td>LN</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18. L.C.</td>
<td>31</td>
<td>NS</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>19. F.R.</td>
<td>42</td>
<td>NS</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>20. L.F.</td>
<td>42</td>
<td>NS</td>
<td>LIVER</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>21. C.S.</td>
<td>54</td>
<td>NS</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>22. V.C.</td>
<td>23</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>23. V.P.</td>
<td>29</td>
<td>NS</td>
<td>MED</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>24. R.L.</td>
<td>20</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>25. F.G.</td>
<td>21</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>26. D.M.</td>
<td>25</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>27. B.M.</td>
<td>23</td>
<td>NS</td>
<td>LN</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>28. Z.L.</td>
<td>26</td>
<td>NS</td>
<td>LN</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>29. S.B.</td>
<td>48</td>
<td>NS</td>
<td>LN</td>
<td>++++</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>30. B.M.</td>
<td>62</td>
<td>NS</td>
<td>LN</td>
<td>++++</td>
<td>+</td>
<td>-</td>
<td>31. B.M.</td>
<td>34</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>32. M.I.</td>
<td>54</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>33. M.M.</td>
<td>70</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>34. B.A.</td>
<td>72</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>35. A.E.</td>
<td>24</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>36. A.L.</td>
<td>31</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>37. A.E.</td>
<td>42</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>38. R.M.</td>
<td>60</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>39. G.L.</td>
<td>83</td>
<td>NS</td>
<td>LN</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: NS, nodular sclerosis; MC, mixed cellularity; LP, lymphocyte predominance; LN, lymph node; MED, mediastinal mass; +, absent; +, rare (<10%); ++, scattered (10% to 20%); ++++, numerous (20% to 50%); ++++, most (50% to 100%); EBV, LMP-1 and/or EBER-1 expression in H-RS; NA, not available; HIV+, patient with acquired immunodeficiency syndrome.
tion of cases, could be regarded as the possible direct consequence of different mechanisms involving either p53-gene mutation or MDM2-mediated p53 inactivation. The immunohistochemically detected accumulation of p53 in H-RS is consistent with both of these hypothetical mechanisms. On the other hand, the accumulation of MDM2 might be a consequence of either MDM2-gene amplification, p53-dependent transcriptional activation of the MDM2 gene, or other unknown mechanisms. According to previous data obtained in human sarcomas, the nuclear immunostaining with I-F2 MoAb can be regarded as the consequence of MDM2 overexpression, although not necessarily caused by amplification of the gene. On the basis of our demonstration of frequent expression of MDM2 in H-RS cells and of recent data suggesting that wild-type p53 is required for transcription of the MDM2 gene, it is possible to speculate that in HD p53 mutations are rare.

The abnormal expression of MDM2 and p53 by H-RS...
cells further supports the notion that they represent the neoplastic component in HD for a number of reasons. First, p53 is a DNA-binding phosphoprotein that exerts a central role as a transcription factor in the control of cell cycle progression and the induction of apoptotic cell death under a variety of conditions such as DNA damage or withdrawal of specific hematopoietic survival factors. Second, the functional loss of p53 can contribute to malignant transformation, as shown by its frequent involvement in human malignancy. The majority of p53 mutations in cancer are of the missense type, producing p53 proteins with longer half-lives that can be shown by immunohistochemistry as nuclear accumulation. Nevertheless, this is not always the case, because in some lymphomas p53 abnormal immunostaining has been shown also in absence of gene mutation. Finally, MDM2 immunostaining of H-RS cells can be considered a further confirmation of their neoplastic nature, because normal lymphoid cells rarely show overexpression in control samples. In addition, the asymmetrical distribution of MDM2 and p53 in multinucleated H-RS can be considered to be evidence of a severely disordered cell cycle in these cells, also in cases with low numbers of H-RS expressing p53 and/or MDM2.

The possible role of EBV proteins in the deregulation of the molecular control of cell proliferation and/or surviving apoptosis deserves a comment. In our series, a proportion of cases exhibited clear molecular signs of latent infection, but a clear correlation with either MDM2 or p53 could not be observed. Nevertheless, it is known that EBV can interfere with the control of cell proliferation through different complex mechanisms. These mechanisms include the direct binding of p53 to virally encoded proteins such as EBNA-50 or an indirect, bcl-2-induced, blockage of p53-dependent apoptosis. In turn, wild-type p53 can promote viral latency in H-RS cells by the binding of p53 with BZLF1 immediate-early transactivator, a protein that is able to control the switch between EBV latency and replication. Accordingly, H-RS cells expressing BZLF1 protein are extremely rare in HD and LMP1 antigen expression is frequent in HD cases harbouring EBV infection, always confined to H-RS cells.

In conclusion, our data show that an abnormal accumulation of MDM2 and p53 proteins occur in HD, raising the question of their possible pathogenetic role in this disease.

REFERENCES


16. Lane DP, Crawford LV: T antigen is bound to a host protein in SV40-transformed cells. Nature 278:261, 1979


Abnormal expression of the p53-binding protein MDM2 in Hodgkin's disease

M Chilosi, C Doglioni, F Menestrina, L Montagna, A Rigo, M Lestani, M Barbareschi, A Scarpa, GM Mariuzzi and G Pizzolo