Expression of the Novel Intermediate Filament-Associated Protein Restin in Hodgkin’s Disease and Anaplastic Large-Cell Lymphoma

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In this study, the expression of the novel intermediate filament protein Restin in human tissues was analyzed. Restin expression was studied by immunohistochemistry using polyclonal and monoclonal antibodies. Restin was not detected in normal tissues, a range of B- and T-cell non-Hodgkin’s lymphomas, and nonlymphoid tumors. However, Restin was present in Reed-Sternberg cells and variants thereof in Hodgkin’s disease, with the exception of the lymphocyte-predominant, paragranuloma subtype. Restin was also highly expressed in anaplastic large-cell lymphoma (so-called Ki-1 lymphoma). As expected, Restin was also expressed in Hodgkin cell lines L428, L428KSA, Co, and KM-H2 and the anaplastic large-cell lymphoma cell line Karpas 299, which was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting, as well as Northern blotting. The presence of Restin in both Hodgkin’s disease and anaplastic large-cell lymphoma is intriguing and might indicate a role of this structural protein in the pathogenesis of both conditions.

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MATERIALS AND METHODS

Tissue samples. The study included tissue samples of 41 cases of Hodgkin’s disease (HD), of which 4 were diagnosed as lymphocyte predominant, nodular subtype; 4 as lymphocyte predominant, diffuse subtype; 22 nodular sclerosis; 9 mixed cellularity; and 2 lymphocyte depletion HD. The study also included six cases of anaplastic large-cell lymphoma (LCL), 14 cases of B-cell non-Hodgkin’s lymphoma (NHL), and nine cases of T-cell NHL, covering most NHL subtypes. In addition, a range of other neoplastic conditions and normal tissues were screened: two samples of normal skin, three of reactive lymph node, two of thyroid, two of thymus, three of liver, four of malignant melanoma, three of malignant fibrous histiocytoma, one of alveolar soft part sarcoma, one of adenocarcinoma of colon, two of squamous carcinoma of the skin, three of hepatocellular carcinoma, and one small-cell carcinoma of the lung. All tissue samples were fixed in B5 and part was snap-frozen in liquid nitrogen-cooled isopentane. The B5-fixed samples were processed for routine morphologic examination. The frozen tissue samples were stored at −70°C until used for immunohistochemistry.

Cell lines and tissue culture. Hodgkin cell lines L428 and L428KSA have been characterized previously.7 Hodgkin cell lines Co and KM-H2 were given, respectively, by D. Jones (Southampton, UK) and S. Fukuhara (Kyoto, Japan).8,9 Anaplastic LCL cell line Karpas 299 was provided by A. Karpas (London, UK).10

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cell lines were all grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS).

Human fetal skin fibroblasts were cultured in DME-F12 (50/50) medium supplemented with 10% FCS.

**Immunohistochemistry.** Four-micrometer cryostat sections of human tissues, cytosin preparations of the cell lines, and tissue slides onto which fibroblasts were grown were used. Acetone was used as a fixative. Restin was detected using a polyclonal rabbit antibody (MRP-70) as well as three monoclonal mouse antibodies (culture supernatants 16-2E6E5, 16-3E2B8, and 16-2F4.D8). Before the application of the antibody, the sections were incubated for 10 minutes with 10% normal swine serum as a blocking reagent. The polyclonal antibody was used diluted 1:100. The monoclonal antibodies (MoAbs) were used undiluted. For immunodetection, either an alkaline phosphatase-conjugated ABC complex (Dakopatts, Glostrup, Denmark) was used after incubation with a rabbit antiserum antibody (Dakopatts), or an APAAP complex (Dakopatts) was used after incubation with a rabbit antiserum antibody (Dakopatts). The color reaction was developed with fast red and naphtol-ASMX-phosphate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Cell lysates were prepared as follows. Cells were washed two times in phosphate-buffered saline (PBS) and 1 x 10^6 cells were resuspended in 50 mmol/L Tris buffer containing 1 mmol/L EDTA, 1 mmol/L iodoacetamide, 1 mg/mL pepstatin, and 1,000 U/mL aprotinin. The cells were briefly sonicated and DNAse I (Sigma Chemical Co, St Louis, MO) was added to a concentration of 0.5 mg/mL. The cell lysate was incubated for 15 minutes at 37°C. SDS and β-mercaptoethanol were added to a final concentration of 1% and the sample was boiled for 5 minutes.

SDS-PAGE (7% polyacrylamide) and transfer of proteins to nitrocellulose filters were performed as described previously. After inactivation with PBS casein, the blots were incubated with the rabbit polyclonal MRP-70 (diluted 1:500) or mouse monoclonal 16-2E6E5 (culture supernatant, diluted 1:5) anti-Restin antibodies. After washing, the blots were incubated with the appropriate peroxidase-conjugated secondary antibody, either swine antirabbit IgG or rabbit antimouse IgG (Dakopatts). After washing, the blot was developed with 3,3' diaminobenzidine and H₂O₂.

**Northern blotting.** RNA was isolated from human fibroblasts, L428KSA cells, and Karpas 299 cells using an RNA isolation kit (Stratagene, La Jolla, CA). The instructions of the manufacturer were followed. Total RNA samples (5 µg) were electrophoresed through 1% agarose-formaldehyde gels and blotted onto Hybond-N membranes (Amersham, Buckinghamshire, UK). Hybridization was performed using the entire coding region of the Restin cDNA, which was 32P-labeled using a random prime labeling kit (Boehringer Mannheim, Mannheim, Germany). Hybridization was conducted in 50% formamide, 5x SSPE, 0.5% SDS, 5x Denhardts, 200 µg/mL salmon sperm DNA, and 10% dextran sulfate for 16 hours at 42°C. Washes were performed in 0.3 x SSPE/0.5% SDS at 60°C.

**RESULTS**

Immunohistochemistry. Similar results were obtained with the polyclonal rabbit or the monoclonal mouse anti-Restin antibodies (summarized in Table 1). Restin expression was only seen in the cytoplasm. L428, L428KSA, Co, KM-H2, and Karpas 299 cells expressed Restin (Fig 1). Restin was not detected in cultured fibroblasts. In the HD tissues, Restin was detected in typical and variant Reed-Sternberg cells (collectively called Hodgkin cells) (Fig 2).

The number of Hodgkin cells that stained for Restin ranged from 50% to 100% within one section. In the Restin-positive anaplastic LCL cases, all malignant cells were stained (Fig 3). No detectable expression was seen in normal tissues, NHLS other than anaplastic LCL, and other neoplastic conditions.

**SDS-PAGE and Western blotting.** A single band of 160 Kd was observed in all Hodgkin cell lines, whereas an additional band of 135 Kd was seen in the anaplastic LCL cell line Karpas 299 (Fig 4). Restin was not detected in fibroblast extracts (data not shown). Comparable results were obtained with the polyclonal rabbit and the monoclonal mouse anti-Restin antibodies.

**Northern blotting.** Northern blot analysis of Restin messenger RNA (mRNA) expression showed the presence of the 6-kb and 4.2-kb transcripts in both L428KSA and Karpas 299 cell lines, but no Restin transcript was detected in human fibroblasts (Fig 5).

**DISCUSSION**

Restin was found to be expressed in Reed-Sternberg cells of HD and the malignant cells of anaplastic LCL, but not in the other tissues tested, including normal tissues. Western blotting and Northern blotting on cell lines confirmed the expression of Restin in HD and anaplastic LCL. On Western blot, anaplastic LCL cell line Karpas 299 shows a band of 135 Kd in addition to the 160-Kd band observed in the Hodgkin cell lines. The existence of protein isoforms of Restin as a result of alternative splicing is excluded because both 6-kb and 4.2-kb transcripts contain the entire open reading frame (unpublished data). Differences in posttranslational modifications might explain the two bands observed on Western blot and are currently being investigated. Northern blotting with a Restin cDNA probe covering the entire coding region showed a band of 6 kb and one of 4.2 kb in the L428KSA Hodgkin cell line and the Karpas 299 anaplastic LCL cell line. These two bands may be the result of the use of different polyadenylation sites, as predicted from the Restin cDNA sequence.

The pattern of Restin expression in human tissues is not surprising because Restin was cloned from the U-937 cell line, which is derived from a patient with malignant
Fig 1. L428KSA cells stained with monoclonal anti-Restin antibody 16-2E6E5 using the APAAP technique (original magnification, ×420).

Fig 2. Reed-Sternberg cells in a case of nodular sclerosing HD stained with monoclonal anti-Restin antibody 16-2E6E5 using the APAAP technique (original magnification, ×200; inset, ×420).

Fig 3. Anaplastic LCL (Ki-1 lymphoma) stained with monoclonal anti-Restin antibody 16-2E6E5 using the APAAP technique (original magnification, ×100).
Fig 4. Western blot on Hodgkin cell lines L428KSA (lane 1), L428 (lane 2), Co (lane 3), KM-H2 (lane 4), and anaplastic LCL cell line Karpos 299 (lane 5) reacted with monoclonal anti-Restin antibody 16-2E6E5.

Histiocytosis, a clinical entity closely related to anaplastic large cell lymphoma or Ki-1 lymphoma. To date, there are few studies on IF protein or IFAP expression in lymphomas. Expression of vimentin, the intermediate filament of mesenchymal tissues, has been assumed to be expressed by all leukocytes and leukocyte-derived malignancies. Recently, Gustmann et al., using a panel of MoAbs demonstrated vimentin expression in the majority of Hodgkin’s lymphomas and NHLs, including anaplastic LCL. Aberrant keratin expression was found in a number of anaplastic LCL cases. Other investigators did not confirm these findings and showed that vimentin is expressed only in a minority of normal lymphocytes and is absent in a substantial number of Hodgkin’s lymphomas and NHLs. However, only one monoclonal antivimentin antibody was used in these studies, which may explain the limited reactivity. No data have been reported on IFAP expression in lymphomas.

The expression of the IFAP Restin in both HD and anaplastic LCL, but not in other NHLs, is intriguing. Although HD and anaplastic LCL are different clinically and the cell of origin in HD is not known as opposed to the established lymphoid origin of most cases of anaplastic LCL, both conditions share the expression of leukocyte activation antigens such as CD30. Interestingly, CD30-expressing activated cells such as Epstein-Barr virus (EBV)-transformed lymphoblastoid cells also express Restin. Taken together, these data suggest that Restin plays a role in leukocyte activation, and may assume a similar function in tumors expressing an activated leukocyte phenotype. Whereas membranous activation antigens such as CD30 possibly represent growth regulating receptors, which may be important in growth control of the malignant cells in HD and anaplastic LCL, the function of the novel IFAP Restin is not clear. However, the sequence of Restin may provide a clue. The heptad repeats and the lack of prolines in long stretches of the Restin sequence suggests the ability of the protein to form homodimeric complexes in which coiled coil configurations are present. These are features of structural proteins. Interestingly, the gene products of MCC (mutated in colorectal cancer) and APC (adenomatous polyposin coli), genes involved in colorectal adenomas and carcinomas, also display these characteristics. Such structural proteins may change cell shape and result in the derepression of a mitogenic signal, and thus function in cell activation or malignant transformation.

Restin is not expressed in paragranuloma HD. It has become clear that this subtype of HD is akin to low-grade NHL of B-cell type; the L/H cell, the Hodgkin cell variant in paragranuloma HD, expresses B-cell antigens but no activation antigens. Ig light-chain restriction has recently been demonstrated and paragranuloma HD can progress to a large-cell NHL of B-cell type. Therefore, it is not surprising that, as in the other NHL, Restin is not

Fig 5. Northern blot analysis of Restin mRNA expression. Total RNA (5 µg) from L428KSA cells (A), Karpos 299 cells (B), and human fibroblasts (C). The blot was exposed for 36 hours.
expressed in paragranuloma HD. Restin was also not detected in a small number of HD cases other than lymphocyte-predominant subtype and in one case of anaplastic LCL. This might indicate that HD as well as anaplastic LCL are heterogeneous and are syndromes rather than entities, as has already been suggested.43 In conclusion, we have demonstrated the selective expression of the novel intermediate filament-associated protein Restin in HD and anaplastic LCL, but not in normal tissues, other NHLs, or other neoplastic conditions tested. The role of Restin in the pathogenesis of HD and anaplastic LCL has yet to be studied.

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