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Variable Expression of Human Myeloid Specific Nuclear Antigen MNDA in Monocyte Lineage Cells in Atherosclerosis

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Abstract *MNDA* (human myeloid nuclear differentiation antigen) is expressed in specific lineages of hematopoietic cells and most notably at high levels in macrophages at sites of inflammation. MNDA and related proteins appear to modulate the activity of transcription factors and in some cases have a role in mediating cell death. The expression of *MNDA* was characterized in normal and diseased human aorta. MNDA positive cells double labeled for CD68 in all tissue examined. Twenty percent of normal aortas were negative or contained rare MNDA positive cells while other normal aorta contained more frequent positive cells. In atherosclerotic aorta, the number of MNDA positive cells increased with progression of disease. In normal and early lesions, MNDA positive cells adjacent to the endothelium generally displayed a strong MNDA reactivity associated with small amount of CD68 reactive cytoplasm. In the same sections, MNDA positive cells at increasing distances from the endothelium displayed lower MNDA reactivity and were associated with larger amounts of CD68 reactive cytoplasm. Foam cells in fatty streaks exhibited MNDA reactivity that ranged from strong to weak or negative. In advanced lesions, cells in the shoulder and those in fibrous tissue surrounding an atheroma were highly reactive for MNDA. However, only a fraction of the CD68 positive foam cells near the lipid core under the cap and shoulder contained MNDA reactivity. The variation in *MNDA* expression appeared to change with phenotypic specialization of monocytes in atherosclerosis consistent with its association with inflammation and suspected roles in regulating gene expression or in mediating cell death. J. Cell. Biochem. 95: 293–301, 2005.

Key words: monocytes; macrophages; atherosclerosis; *MNDA*; immunohistochemistry

The involvement of macrophages in atherosclerosis appears to begin with adhesion of monocytes to the endothelium, which is followed by migration between endothelial cells into the intima and then transformation into tissue macrophages. Examination of a number of features of macrophages suggests the presence of diverse functional subsets in atherosclerosis [Hakkinen et al., 2000; Sugiyama et al., 2001]. The ingestion of lipoproteins is believed to transform macrophages into foam cells. A recent survey of changes in the expression of thousands of genes associated with the in vitro transformation of cultured macrophages to foam cells was completed with the purpose of identifying new genes with roles in the pathogenesis of atherosclerosis and provide target genes whose expression could be altered in efforts to reduce the morbidity associated with the disease [Shiffman et al., 2000]. The expression of over 200 genes was significantly altered over 4 days of ingesting oxidized low-density lipoprotein. *MNDA* was the third most highly down regulated feature identified [Shiffman et al., 2000].

The *MNDA* (human myeloid nuclear differentiation antigen) is a member of a family of interferon-regulated genes (*IFI 200* family) identified in human and mouse [Burrus et al., 1992; Briggs et al., 1994a; Landolfo et al., 1998]. The hematopoietic lineage restricted pattern of expression, that was the basis for the identification of *MNDA*, is unique characteristic of genes in this family [Goldberger et al., 1984, 1986]. A recent comprehensive examination of normal

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and neoplastic tissues confirmed the hematopoietic cell restricted patterns of *MNDA* expression [Miranda et al., 1999]. In addition, this analysis showed that while MNDA was detected in myeloblasts and all later stage myelomonocytic cells, including peripheral blood monocytes and granulocytes, MNDA was generally not detected in tissue histiocytes except at sites of inflammation [Miranda et al., 1999]. MNDA has been implicated in regulating the expression of *DLK1*, a notch related gene that is involved in hematopoiesis [Doggett et al., 2002; Ohno et al., 2002]. Those findings are consistent with the lineage specific pattern of *MNDA* expression and with the suspected role of *IFI200* genes as modulators of transcription factor function and factors mediating apoptosis [Landolfo et al., 1998].

The critical role of monocytes in atherosclerosis is related to the development of specialized functional forms that occur at specific sites during each of the stages of the disease. Normal vessels and atherosclerotic lesions ranging from minimal to advanced or complicated were evaluated for *MNDA* expression to determine if expressing cells were present, and to test the hypothesis that *MNDA* expression changes with the differentiation and functional specialization of monocytes or with cell death.

MATERIALS AND METHODS

Human Tissues

Specimens of aorta were collected as part of the "Pathobiological Determinants of Atherosclerosis in Youth" research program [PDAY Research Group, 1993]. Samples of mid abdominal aorta, 15 mm distal to the renal artery orifices, were from black and white males and females aged 15–34 years who died of trauma suddenly and unexpectedly, and in whom the postmortem interval was less than 10 h. Additional abdominal aorta samples were collected from subjects aged 40–80 years who underwent postmortem examination at Vanderbilt University Medical Center in which the postmortem interval was also less than 10 h. Formalin-fixed samples were processed for light microscopy and paraffin-embedded sections were stained with hematoxylin–eosin and Movat pentachrome stains [Luna, 1968]. Atherosclerotic lesions were classified by criteria defined by the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association as: (1) type I (isolated or small groups of foam cells in areas of adaptive intimal thickening and atherosclerosis-prone areas); (2) type II (fatty streaks, consisting of collections of intimal foam cells); (3) type III (intermediate, or transition lesions [preatheromas] with intimal foam cells and extracellular lipid); (4) type IV (atheromas, with extracellular lipid that occupied a well-defined and extensive area within the intima); and (5) type V (fibroatheromas, with a prominent fibrous tissue component and/or calcification). Type VI (complicated) lesions are type IV and V lesions with surface disruption, hematoma or thrombosis. Samples were embedded longitudinally and the most advanced lesion within any given segment was used to classify the lesion and was the portion of the aorta that was analyzed.

Immunohistochemical Studies

Four-micrometer thick paraffin-embedded tissue sections were prepared for antibody staining as previously described [Miranda et al., 1999]. Slides from each case were exposed for 1 h to a 1:1,000 dilution of 3C1 rat monoclonal antibody (anti-MNDA) [Xie et al., 1998] or mouse monoclonal antibodies (1:100) against smooth muscle actin, CD68, or CD3 (Dako Co., Carpinteria, CA). Reagent control slides were included and an isotype-matched irrelevant antibody (#U3254, Sigma, St. Louis, MO) was used to control for anti-MNDA reactivity. An avidin–biotin–peroxidase complex (Vector Laboratories, Inc., Burlingame, CA) specific for each antibody was used. Antibody binding was visualized using 3,3′-diaminobenzidine (liquid DAB, BioGenex, San Ramon, CA) and when slides were double labeled the second antibody reaction was detected using 3-amino-9-ethylcarbazole (BioGenex). Prior to incubation with the first and second primary antibodies, sections were exposed or re-exposed to 3% H_2O_2 in methanol for 20 min. in order to quench endogenous or residual peroxidase activity. Slides were counter stained with hematoxylin (BioGenex) after completing immunochemical staining. The same areas of each section were photographed after completing each staining reaction.

Blood monocytes and granulocytes found in small vessels in the adventitia of the arteries provided a positive internal control for MNDA reactivity. All cases were interpreted by each of the authors. The categories of MNDA positive

reactivity within the intima of sections were as follows: (1) frequent—more than 1 positive cell per 70 μm length of vessel; (2) occasional—less than 1 positive cell per 70 μm length of vessel wall, but more than one positive cell per 700 μm length of vessel; and (3) rare—less than one positive cell per 700 μm length of vessel. Diffuse indicates the same pattern of staining over most of the section and focal indicates localized sites of positive staining.

RESULTS

The specificity of the rat monoclonal antibody used in this work has been demonstrated repeatedly using a variety of immunochemical assays [Hudson et al., 1988; Briggs et al., 1994a; Miranda et al., 1999]. In addition, when the antibody was used in an affinity matrix for protein purification, over half of the MNDA amino acid sequence was obtained using conventional N-terminal sequencing [Briggs et al., 1992; Burrus et al., 1992]. Sections of artery containing intima of normal thickness and without evidence of foam cells were considered normal. The number of MNDA positive cells present in the intima of normal artery varied from none to frequent (Table I). Out of 15 normal cases only three were negative or contained rare MNDA positive cells (data not shown). Twelve cases contained more frequent MNDA positive cells and these double labeled for CD68 (Fig. 1A,B).

Seven cases with type I lesions contained frequent MNDA reactive nuclei (Table I). In type I lesions, MNDA reactive nuclei were generally darker in focal areas that were not occupied by foam cells (Fig. 2A). In cases where cells contained large amounts of CD68 reactive material as in foam cells (Fig. 2B,C), MNDA activity varied and in some CD68 reactive cells no MNDA reactivity was detected.

TABLE I. Evaluation of Human Myeloid Nuclear Differentiation Antigen (MNDA) Reactivity in Early Atherosclerotic Lesions

Cases	N	Pattern of reactivity
Normal	1	Negative
	2	Rare
	1	Diffuse occasional
	2	Focal frequent
	9	Diffuse frequent
	Total 15	
Type I	3	Focal frequent
	4	Diffuse frequent
	Total 7	
Type II	1	Focal frequent
	8	Diffuse frequent
	Total 9	
Type III	8	Diffuse frequent
	Total 8	
Type IV	8	Diffuse frequent
	Total 8	
Type V	7	Focal frequent
	2	Diffuse frequent
	Total 9	

All nine cases classified with type II lesions (fatty streaks) contained frequent MNDA reactive nuclei (Table I). Vessels in these cases had uniformly thickened intima and MNDA positive cells near the endothelium reacted strongly, while MNDA positive cells located deep within the intima showed less intense reactivity (data not shown). MNDA positive cells double labeled for CD68, but those adjacent to the endothelium had scant cytoplasm, and weakly MNDA reactive cells within the intima had more cytoplasm that was CD68 reactive. In many cases, the cells that contained large amounts of CD68 positivity appeared to be clusters of foam cells with vacuolated cytoplasm. Additional double labeling experiments showed that MNDA reactive cells uniformly double label for CD68 and not for smooth muscle actin or CD3 (data not shown).

Cases with type III lesions (intermediate or transition lesions/preatheromas) contained small pools of extra cellular lipid and had frequent MNDA positive cells. As in type I and II lesions, a gradient of MNDA reactivity was observed with darkest stained cells nearest the endothelium (Fig. 3A,B). CD68 positive foam cells negative or weakly reactive for MNDA, as observed in type II lesions, were also observed in cases with type III lesions (Fig. 3B).

The eight type IV lesions consisted of atheromas with well defined shoulder regions (Fig. 4A) and fibrous caps (Fig. 4B) that contained numerous cells strongly reactive for MNDA (Table I). However, in areas adjacent to the acellular lipid core near the shoulder or cap, some CD68 positive foam cells were MNDA negative (Fig. 4C,D).

In type V fibroatheroma lesions, the thickened fibrous cap contained frequent cells strongly positive for MNDA (Table I). However, the density of MNDA positive cells was noticeably lower than that observed in the cap of type IV lesions (data not shown). Type V lesions contained cells with variable levels of MNDA reactivity; strong and weakly reactive cells were distributed throughout the cap and intima and did not exhibit a gradient of activity related to distance from the endothelium as was observed

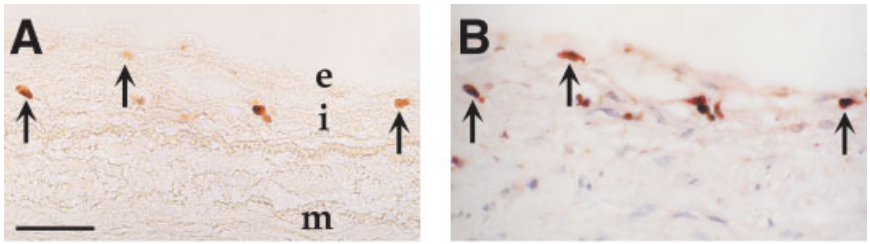

Fig. 1. Immunohistochemistry of normal aorta with diffuse frequent human myeloid nuclear differentiation antigen (MNDA) reactivity. **A**: Section after immunoperoxidase staining for MNDA with DAB substrate (brown reaction product). **B**: Same area of section after additional immunohistochemical staining for CD68 with AEC substrate (red reaction product) followed by staining with hematoxylin. The same cells in (A and B) show MNDA and CD68 positivity (arrows). There appears to be an inverse relationship between amount of CD68 reactivity and the intensity of MNDA staining. e, endothelial cells; i, intima; m, media. Bar = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in normal aorta and in type I, II, III, and type IV lesions (data not shown). While clusters of CD68 positive foam cells were infrequent, they also contained a mix of MNDA positive and negative cells (data not shown).

In five cases with advanced/complex type VI lesions, the dense fibrous layer overlying the atheroma contained mostly cells with dark MNDA reactive nuclei (diffuse frequent), and these cells generally contained scant cytoplasmic CD68 reactivity (data not shown). CD68 positive foam cells within the atheroma contained strong, weak or no MNDA reactivity (Fig. 5A,B). Frequently, the largest foam cells were MNDA negative and the smaller foam cells were generally positive, and many of these cells were strongly reactive (Fig. 5). The level of MNDA reactivity appeared as a gradient becoming less intense in foam cells adjacent to the lipid deposits in the acellular core. Often smaller tightly packed foam cells that were near the fibrous cap were strongly MNDA positive (Fig. 5A). The shoulder region of atheromas contained large numbers of cells strongly reactive for MNDA that were associated with CD68 reactive material (data not shown). Additional cells in the shoulder contained nuclei negative for MNDA and these cells were usually a mix of CD68 positive and negative. Cells that were not CD68 positive were very likely MNDA negative as they were probably not derived from the monocyte lineage. Additional experiments with these cases showed that MNDA did not double label smooth muscle actin or CD3 positive cells.

DISCUSSION

Previous experiments established that MNDA expression in tissue histiocytes varied from none (e.g., Kupffer cells and microglia) to high levels in histiocytes at sites of chronic inflammation [Miranda et al., 1999]. A similar variation in the level of MNDA expression was demonstrated in atherosclerosis. In early stage lesions, a gradation in MNDA level correlated with distance of CD68 positive cells away from the endothelium within the intima. As the level

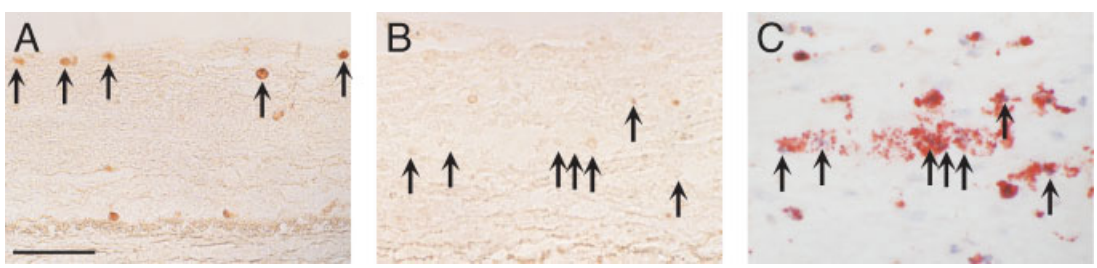

Fig. 2. Immunohistochemistry of a case with type I lesion and focal frequent MNDA reactive cells. **A**: Section after immunoperoxidase staining for MNDA with DAB substrate. The area shown in (A) is devoid of foam cells and MNDA positive cells (arrows) have small amounts of associated CD68 reactivity. **B**: Second area of the same section as in (A) after immunohistochemical staining of MNDA with DAB substrate. **C**: Same area as in (B) after additional staining for CD68 with AEC substrate followed by staining with hematoxylin. Scattered foam cells (arrows) show little or no reactivity for MNDA. Bar = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

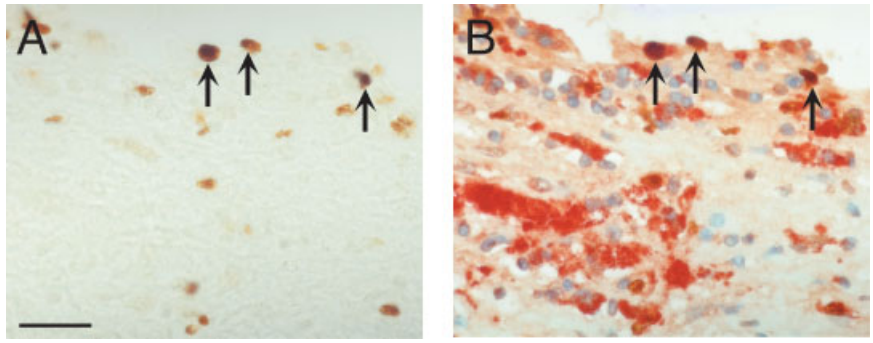

Fig. 3. Immunohistochemistry of a case with type III lesion and diffuse frequent MNDA reactive cells. **A**: Section after immunoperoxidase staining for MNDA with DAB substrate. **B**: Same area of section in (A) after additional immunohistochemical staining for CD68 with AEC substrate followed by hematoxylin staining. Cells strongly reactive for MNDA tend to localize adjacent to the endothelium (arrows). Cells within clusters of foam cells (**lower left** in B) tend to be less reactive or non-reactive for MNDA. Numerous hematoxylin staining nuclei in that area are not MNDA reactive (compare B to A). Bar = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of MNDA reactivity dropped in cells more distant from the endothelial surface, the amount of CD68 reactive cytoplasm progressively increased in double labeled cells appearing to coincide with the transition to macrophages. Clusters of CD68 positive foam cells in type II and III early stage lesions also showed low levels of MNDA reactivity or none. In type IV and type V, atheromas and in complex lesions, CD68 positive foam cells also displayed a variable level of

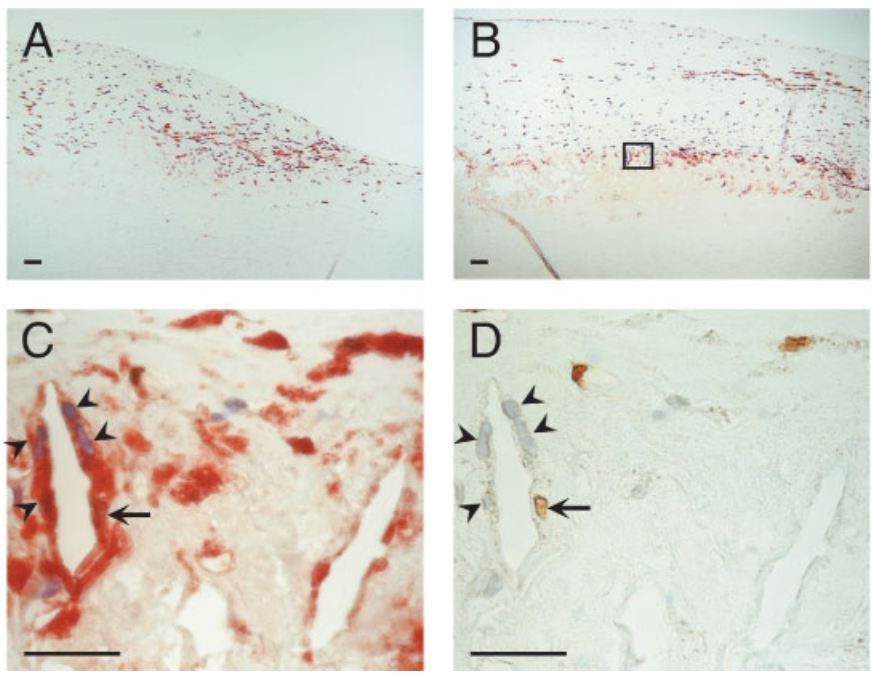

Fig. 4. Immunohistochemistry of a case with type IV lesion and diffuse frequent MNDA reactive cells. **A**: Shoulder region of an atheroma after immunoperoxidase staining for MNDA with DAB substrate, CD68 with AEC substrate followed by hematoxylin staining. Bar = 10 μm. **B**: Fibrous cap of atheroma following staining as in (A). Bar = 10 μm. **C**: Region between fibrous cap and acellular lipid core (area in box in B). Immunoperoxidase staining for MNDA with DAB substrate and CD68 with AEC substrate followed by hematoxylin staining. Bar = 10 μm. **D**: Same region in (C) after destaining CD68 AEC reaction product with ethanol. Bar = 10 μm. Examination of cells surrounding the cholesterol crystal in (C) shows a uniform CD68 positivity as well as hematoxylin staining nuclei. After destaining the AEC generated reaction product (D), 1 weak MNDA reactive cell (arrow) is apparent while the remaining cells are MNDA negative (arrowheads). The hematoxylin staining demonstrates that the MNDA negative cells contain nuclei. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

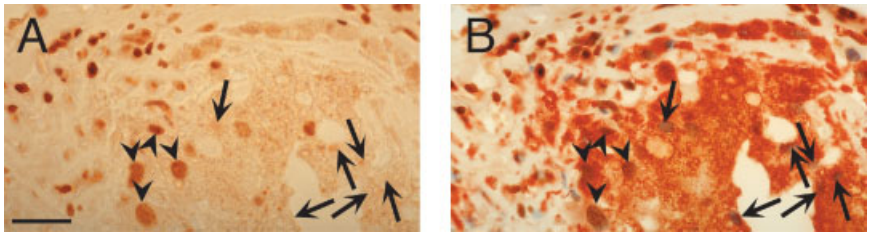

Fig. 5. Immunohistochemistry of a case with type VI complicated lesion and diffuse frequent MNDA reactive cells. **A**: Section of a region between fibrous cap (**top**) and acellular lipid core (**lower right**) after immunoperoxidase staining for MNDA with DAB substrate. **B**: Same area of section in (A) after additional immunohistochemical staining for CD68 with AEC substrate followed by hematoxylin staining. A number of nuclei staining with hematoxylin in (B) in CD68 positive cells near the acellular lipid core are non-reactive for MNDA in (A) (arrows). Other CD68 positive cells contain strong MNDA positive nuclei (arrowheads). Bar = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MNDA reactivity, ranging from high in cells adjacent to the fibrous cap, to none detected in foam cells located adjacent to the acellular lipid core. MNDA positive cells were always colabeled with CD68 and not smooth muscle actin or CD3.

The findings were consistent with MNDA marking monocytes, macrophages, and macrophage foam cells in atherosclerotic tissue and not lymphocytes, smooth muscle cells, or vascular dendritic cells which are CD68 negative [Bobryshev and Lord, 1998]. The fibrous cap and shoulder regions associated with type IV and type VI complex lesions contain monocytes and macrophages that uniformly expressed high levels of MNDA. These situations appear to resemble sites of inflammation in other tissues where MNDA expression was uniformly high in macrophages and foreign body giant cells [Miranda et al., 1999]. Macrophages located in the fibrous cap or in the shoulder region of atheromas (type IV and VI complex lesions) express a high level of MNDA. The question of whether this high level of MNDA represents an upregulation after drop in expression following extravasation of the monocyte or a persistent high level of expression as observed in monocytes adjacent to the endothelium in normal aorta, and in type I, II, and III lesions could not be resolved. The uniform high level of MNDA expression in CD68 positive cells in the shoulder and fibrous cap surrounding the atheroma in advanced lesions would be consistent with MNDA expression in macrophages found at sites of chronic inflammation. The observation of a gradation of MNDA expression in CD68 positive cells in early lesions (high in cells near the endothelium and progressively lower levels as cells approach the elastic or medial layer) suggests that the intima is not as pro-inflammatory at early stages of atherosclerosis as in complex lesions. Also, cells containing a uniformly high level of MNDA reactivity, in the fibrous cap and shoulder of advanced lesions coincide with the location of the highest levels of pro-inflammatory cytokines [Frostgard et al., 1999].

The phenotypic differences between macrophages/foam cells located in the fibrous cap and shoulder with foam cells located between the acellular lipid core and the fibrous cap is reflected in the patterns of expression of matrix metalloproteinases [Galis et al., 1994; Halpert et al., 1996]. The similarity of MNDA and platelet activating factor receptor (PAF-R) expression in monocyte/macrophages and foam cells of atherosclerotic lesions is also striking [Brocheriou et al., 2000]. Monocytes adjacent to the endothelium were strongly reactive for PAF-R and MNDA. Small macrophages in the intima showed heterogeneous staining and foam cells were weakly stained or even negative for these antigens. The similarities in the pattern of MNDA to that of other genes in monocyte/macrophage/foam cells within atherosclerotic lesions suggests an association with changes in cellular physiology that accompanies progression of the disease. It is tempting to speculate that the presence of MNDA expression in cells within the shoulder region of atheromas could serve as a marker of and/or play a role in plaque rupture leading to complicated lesions with hemorrhage and thrombosis, and thus will be an important area for future investigation.

The level of MNDA in peripheral blood monocytes is high and is comparable to the level in cell lines such as HL-60 and U937 [Briggs et al.,

1994c]. However, isolated monocytes maintained in culture for more than 6 days showed a progressive loss of MNDA as the cells assumed features of macrophages [Briggs et al., 1994c]. The progressive loss of MNDA reactivity in monocytes and macrophages in the intima of early stage atherosclerosis might represent the same process taking place in vivo.

Experiments using HL-60 cells in culture showed that the induction of monocyte/macrophage differentiation was also accompanied by a decrease in *MNDA* mRNA to an undetectable level [Briggs et al., 1994b,c]. Interestingly, the level of *MNDA* mRNA in differentiated HL-60 cells or in cultured monocytes was upregulated with appropriate inducers [Briggs et al., 1994b], which is consistent with the observation that activated macrophages associated with inflammation can be induced to express a high level of *MNDA*. In the case of foam cells, the level of *MNDA* showed a delayed drop as foam cells developed in vitro [Shiffman et al., 2000]. The loss of MNDA in foam cells documented in this study in atheromas appears to represent the same phenomenon occurring in vivo. However, in complex lesions, it appears as though the environment resembles that of an inflammation and both foam cells and macrophages express a uniformly high level of MNDA. Under these conditions, the absence of MNDA in foam cells adjacent to the acellular lipid core is in stark contrast to the surrounding tissue and indicates that MNDA is targeted for degradation with cell death.

The biological significance of the changes in MNDA expression in monocyte derived cells involved in atherosclerosis may be linked to the suspected function of genes in the *IFI200* family. Attempts to establish function of these genes have focused on the characterization of interactions with transcription regulatory proteins and the effects on gene transcription. MNDA and related proteins, specifically mouse p202a and human IFI16, are known to interact with a large number of other nuclear proteins including transcription factors [Johnstone et al., 1998; Xie et al., 1998; Wang et al., 2000]. Both mouse p202a and human IFI16 have been reported to influence promoter activity in transfection experiments, and the p202a has been observed to affect endogenous gene expression [Wang et al., 2000]. The mouse p202a has been reported to inhibit the actions of many transcription regulatory proteins including AP-1 and NF-κB protein subunits [Datta et al., 1998; Wang et al., 2000]. IFI16 binds p53 and stimulates promoter-reporter activity of target p53 regulated sequences [Johnstone et al., 2000].

Ectopic expression of *MNDA* in the non-expressing K562 cell was associated with up-regulated *DLK1*, a gene involved in normal hematopoiesis and specifically macrophage development [Laborda, 2000; Ohno et al., 2002]. The importance of altering *DLK1* expression in differentiation in a number of cell lineages has been documented and participation in the generation of diverse specialized monocytic cells in atherosclerosis would be consistent with its actions in other systems [Laborda, 2000].

An N-terminal region of MNDA was previously found to be essential for homodimerization [Xie et al., 1997]. This N-terminal region of MNDA was recently identified as a DAPIN (domain in apoptosis and interferon response)/pyrin domain [Staub et al., 2001], which is a protein–protein interaction site responsible for protein homodimerization as well as heterodimerization of other proteins with DAPIN domains [Staub et al., 2001]. As observed in MNDA, the DAPIN domain is only found on the N-terminus of a subset of proteins involved in apoptotic and inflammatory signaling pathways. The pyrin protein contains a DAPIN/pyrin domain and is expressed at high levels in granulocytes and monocytes and is upregulated by proinflammatory agents. MNDA has similar expression characteristics. The possibility that MNDA interacts and functions through its DAPIN/pyrin domain with a group of proteins involved in signaling apoptosis suggests an additional area to consider in attempting to elucidate the significance of the changes in MNDA expression observed in atherosclerosis. Specifically loss of MNDA in foam cells adjacent to the acellular lipid core might represent functionally significant changes in cell physiology that correlates to eventual foam cell death at that site as has been characterized previously [Ball et al., 1995]. The present findings are consistent with MNDA function associated with specialized development of monocyte derived cells in atherosclerosis. In addition, MNDA appears to be a target for degradation in the same cells in association with cell death indicating that MNDA may promote cell survival.

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