A macrophage migration inhibitory factor is expressed in the differentiating cells of the eye lens

GRAEME J. WISTOW*†, MICHAEL P. SHAUGHNESSY*, DOUGLAS C. LEE*, JASON HODIN*, AND PEGGY S. ZELENKA‡

*Section on Molecular Structure and Function, and ‡Section on Cellular Differentiation, Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, MD 20892

ABSTRACT   A discrete 10-kDa polypeptide (10K) is expressed from early stages in the embryonic chicken lens. Since this has potential as a marker for lens cell development, chicken 10K and its homologues from mouse and human lenses were identified by protein sequencing and cloning. Surprisingly, lens 10K proteins appear to be identical to a lymphokine, macrophage migration inhibitory factor (MIF), originally identified in activated human T cells. Using microdissection and PCR techniques, we find that expression of 10K/MIF is strongly correlated with cell differentiation in the developing chicken lens. Northern blot analysis shows that 10K/MIF is widely expressed in mouse tissues. These results suggest that proteins with MIF activity may have roles beyond the immune system, perhaps as intercellular messengers or part of the machinery of differentiation itself. Indeed, partial sequence of other small lens proteins identifies another MIF-related protein (MRP8) in calf lens. The relatively abundant expression of MIF in lens may have clinical significance, with the possibility of involvement in ocular inflammations that may follow damage to the lens.

Vertebrate lenses are particularly benign environments for many proteins, allowing the accumulation and retention without turnover of various soluble proteins that may serve as crystallins, the principal determinants of lens refractive index (1–4). Other proteins may also benefit from this environment, becoming moderately abundant in the lenses of some species without achieving the extremely high levels of crystallins. A distinct abundant 10-kDa polypeptide (10K) is present in the developing chicken lenses from early stages. This protein presents interesting possibilities as a marker for lens development. Chicken lens 10K from 11-day embryos was isolated and examined by microsequencing (5). Chicken, mouse, and human lens 10K proteins were then examined by PCR and cDNA cloning.§ Surprisingly, lens 10K proteins appear to be identical to a class of lymphokines with macrophage migration inhibitory factor (MIF) activity.

MATERIALS AND METHODS

Protein Sequence. Soluble lens extracts from 11-day embryonic chicken and newborn calf lenses were separated by SDS/PAGE. Proteins were blotted onto nitrocellulose. Chicken 10K and calf 14-kDa (14K) fractions were excised and examined by microsequencing, by using the facilities of the Harvard Microchemistry Facility. All methods were as described (5).

cDNA Cloning and Sequencing. Primers [5064, CAGGATC-CCGATGTTCA(TC)C(GA)TA(AC)ACACCAA; 5065, TAGTCGACGGT(GATC)GA(GA)TT(GA)TTCCA (GC)CC; containing *Bam*HI and *Sal* I sites, respectively]

were designed from N- and C-terminal chicken peptide sequences, with redundancies suggested by the human T-cell MIF sequence (6). PCR of chicken lens total RNA (7) yielded a specific product that was subcloned and sequenced. This cDNA was then used as a probe to screen 11-day embryonic chicken and newborn mouse lens cDNA libraries. Human lens 10K/MIF sequence was obtained by PCR of total RNA extracted from a pair of human fetal lenses (13.5-week fetal lenses from 1984 stored in liquid nitrogen) by using primers 5064/5065, followed by subcloning and sequencing. Overlapping and 3' untranslated sequences were obtained by PCR with primer 5064 and an oligo(dT)-containing primer. This product was subjected to thermocycle sequencing by using reagents and protocols from Promega.

Northern Blot Analysis. The mouse lens cDNA clone was used as a probe. RNA was extracted, blotted, and hybridized as described (8). The probe was labeled by random-priming using [^{32}P]dATP (Amersham). Hybridization was overnight at 42°C in a formamide-containing buffer, washing was in 0.1× standard saline citrate/1% SDS at 65°C, and exposure was overnight.

PCR Analysis of mRNA Distribution in Chicken Lens. Dissections, RNA extraction, and reverse transcription (RT)–PCR were as described (7). A deleted internal standard (9) was included that was constructed from the chicken 10K/MIF cDNA sequence by using four primers: (*i*) 5986, ATGCCGATGTTCACCATACACAC; (*ii*) 5987, CAATGC-TGTAGAGGCGGACATCTGATCAGG; (*iii*) 5988, CCT-GATCAGATGTCCGCCTCTACAGCATTG; (*iv*) 5989, GTGGAATTGTTCCAGCCCAC. Primers 5987 and 5988 overlap to produce a 30-base deletion in the final product. Primers 5986 and 5989 were used to amplify deleted product and also in the quantitative RT–PCR. Duplicate reactions were repeated three times.

Computer Methods. GenBank and National Biomedical Resource Foundation data bases were searched using SEQFP, SEQF, and SEQFT of the IDEAS package (10). Sequences were aligned using ALIGN (Scientific and Educational Software, State Line, PA).

RESULTS

Lens 10K Sequence. In the developing chicken lens, a distinct polypeptide of ≈10 kDa (10K) (Fig. 1) was observed at all stages examined from 6 to 19 days after fertilization. This polypeptide was isolated by SDS/PAGE separation and
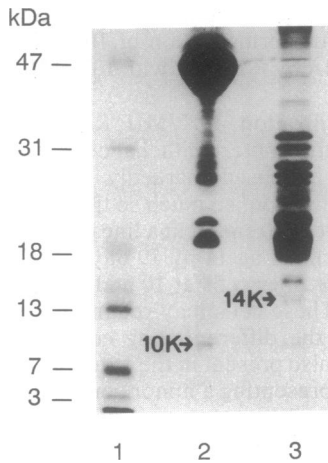
FIG. 1. SDS/PAGE of lens extracts from 11-day embryonic chicken and newborn calf. Gels were slightly overloaded to allow visualization of the chicken 10K (lane 2) and calf 14-kDa (14K; lane 3) proteins. Lane 1 contains molecular mass markers.

subjected to microsequencing. Evidently, chicken lens 10K is unblocked since it yielded N-terminal sequence without the need for proteolysis. Additional sequences were then obtained from tryptic peptides (Fig. 2) (5). When compared with the translated GenBank data base (Release 72), all peptides se-

quenced from the 10K fraction gave close matches with a recently identified human lymphokine, macrophage MIF (6). Interestingly, preliminary analysis of a 14-kDa fraction from cow lens (Fig. 1) yielded an N-terminal sequence (MLTELEKALNSIXDVYH) identical to that of another MIF-related protein, MRP8/cystic fibrosis antigen (11, 12), a Ca^{2+}-binding protein from human lymphocytes.

By using primers derived from chicken peptide and human cDNA sequences, chicken lens 10K was amplified from lens RNA by RT–PCR (13). This PCR fragment was used as a probe to clone 10K from embryonic chicken and 18-day-old mouse lens cDNA libraries (Fig. 2). Full-length coding sequence for chicken lens 10K was obtained, corresponding to a predicted unmodified polypeptide size of 115 amino acids and a molecular mass of 12.4 kDa. Coding and 3' untranslated regions of human lens 10K mRNA were also obtained by RT–PCR from fetal human lens RNA (Fig. 2). Multiple copies of two overlapping human lens 10K/MIF PCR fragments corresponding to bases 77–517 of the clone from human T cells (6) were sequenced, showing identity with the published T-cell sequence except for one base change and the absence of *Pst* I linker. The single base change is a G → A substitution at position 367, causing a Ser-106 → Asn amino acid change.

FIG. 2. cDNA and deduced protein sequences of 10K proteins from embryonic chicken, newborn mouse, and fetal human lens. (*A*) cDNA sequences: T-cell, human T-cell MIF with linker sequences removed; H10K, human lens 10K; M10K, mouse lens 10K; C10K, chicken lens 10K. Chicken and mouse sequences were derived from cDNA clones. Human sequence was derived from two overlapping PCR-derived clones. For comparison the complete deduced sequence of human T-cell MIF is shown with sequence numbers. Overline shows beginning and end of open reading frame. The symbol ! shows single difference between human lens and T-cell sequences. By direct sequencing of PCR fragments, the group of four guanine residues between residues 489 and 492 of the human sequence are slightly ambiguous. (*B*) Deduced protein sequences. Asterisks show peptide sequences obtained for chicken lens 10K protein. Sequence identities shown by the symbol ! indicate difference between human lens and T-cell sequences.
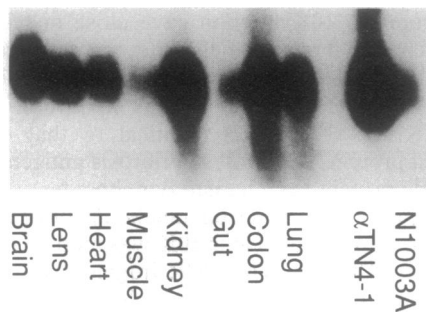
FIG. 3. Northern blot of 3-week-old mouse tissues with a mouse 10K/MIF cDNA probe. RNA samples used were brain, lens, heart, muscle, kidney, gut, colon, lung, and two lens-derived cell lines, αTN4-1 (mouse) (14) and N1003A (rabbit) (15).

Chicken and predicted mouse 10K/MIF sequences also have Asn at this position (Fig. 2). These results suggest that lens 10K and T-cell MIF are products of the same gene.

Localization of 10K/MIF mRNA. Examination of RNA from several tissues of 18-day-old mice shows that 10K/MIF mRNA is widely expressed (Fig. 3). There is apparently no correlation between the expression of 10K/MIF and the level of major histocompatibility complex gene expression in the tissues (8), suggesting that 10K/MIF may have a function distinct from any role in the immune system.

In the lens, populations of quiescent, dividing, differentiating, and terminally differentiated cells are spatially segregated and can be microdissected (7). By using RT–PCR, RNA extracted from microdissected embryonic chicken lenses was examined for the presence of 10K/MIF mRNA (Fig. 4) (9). At several developmental stages, lenses were dissected into inner epithelium, enriched for dividing cells; outer epithelium, enriched for differentiating cells; and differentiated fiber cells. An internal standard for each PCR experiment was engineered by creating a 30-bp deletion (9) in a chicken lens 10K/MIF

cDNA clone. Equal amounts of the standard were added to each PCR mixture and used for normalization of 10K/MIF levels after amplification of RNA by RT–PCR with 10K/MIF-specific primers (Fig. 4).

As early as 6 days after fertilization, 10K/MIF RNA was detected only in the differentiating outer epithelial cells. The strength of 10K/MIF signal increased markedly up to a maximum at 14 days after fertilization, so much so that outer region RNA needed to be diluted to remain in a linear range. After this peak, the signal declined again by 19 days. 10K/MIF RNA also appeared in the fiber cells at 10 and 14 days as the total signal increased. These cells may retain mRNA acquired when they were in the differentiating equatorial population. Some mRNA was also present in the inner region at 10 and 14 days, probably representing a minor component of differentiating cells that becomes more apparent as the amount of 10K/MIF increases. This spatial pattern of expression is different from that of various oncogenes that have been examined in the same system, although it has interesting parallels with the expression of N-*myc* (7, 16).

DISCUSSION

MIF has been observed only in activated T cells (6) from which it is secreted as a lymphokine capable of initiating the inflammatory response in macrophages (6, 17). Here we show that this protein is expressed outside the cells of the immune system and, in particular, in the developing eye lens. The possible function of 10K/MIF in the lens could lie in two general areas. (*i*) It may have the same immune-system-related role as proposed (6). (*ii*) It could have a different or additional role with more general applicability to various cell types.

Although mammals have an embryonic ocular vasculature (18) that is dismantled (probably by macrophages) (19) as the eye matures, embryonic chicken lenses are not vascularized (18) and by 18 days mice have a completely avascular
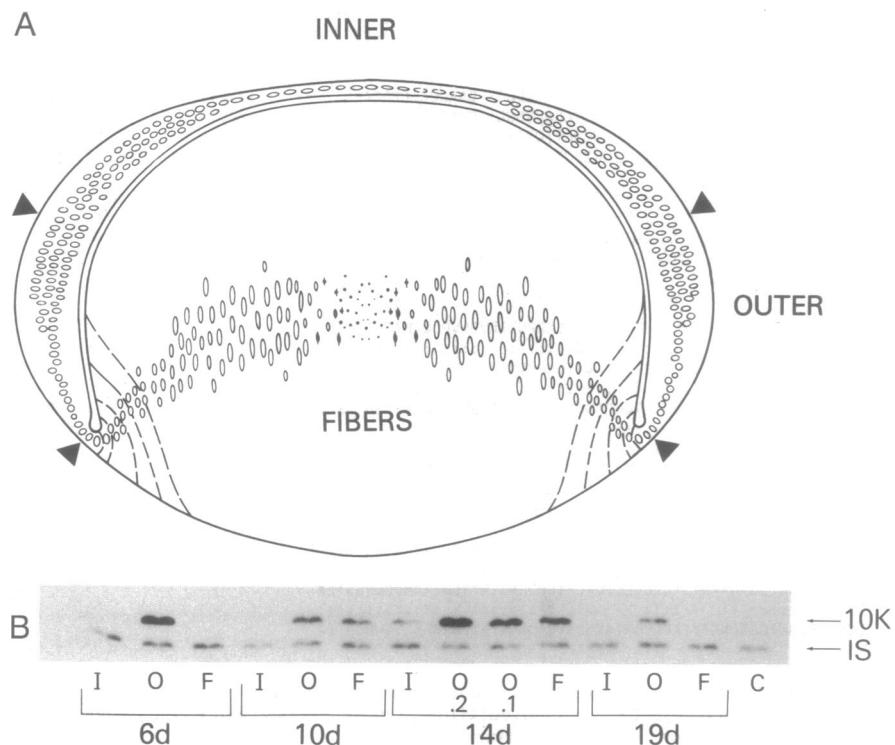

FIG. 4. Expression of 10K/MIF correlates with cellular differentiation in the embryonic chicken lens. (*A*) Scheme for dissection of embryonic chicken lenses. (*B*) Detection of 10K/MIF (10K) mRNA in dissected regions of developing embryonic chicken lenses by using RT–PCR and an internally deleted cDNA standard (IS). Ages shown are days (d) after fertilization. I, inner equatorial epithelium; O, outer equatorial epithelium; F, fibers; C, control with no added RNA. At 14 days, outer region RNA was diluted 1:5 and 1:10 (lanes 0.2 and 0.1, respectively).

encapsulated lens (18). Indeed, the mouse lens is part of the immunologically privileged anterior chamber of the eye and has no class I or class II major histocompatibility complex gene expression (8). The mature lens thus seems to have no direct interaction with the immune system. There are, however, sparse macrophage-related cells called hyalocytes in the vitreous body of the eye (19). Conceivably, 10K/MIF secreted from lens could act upon these cells if it is capable of passing through the lens capsule (which is not yet known). In this way, or directly, 10K/MIF could be involved in the communication between lens and retina, which is important for normal eye development (see ref. 20).

However, 10K/MIF clearly belongs to the class of active polypeptides with assorted growth and mitogenic effects. It has been shown to be secreted and to act remotely on macrophages (6) like many growth factors and cytokines. However, many growth factors and cytokines, including the fibroblast growth factor (21, 22) and interleukin (23) families, may have additional autocrine roles in various tissues. If this is also the case for 10K/MIF, there might be an association between its expression and general processes such as cell growth and differentiation. The lens has particular advantages for examining the relationship between gene expression and cellular differentiation since populations of quiescent, dividing, differentiating, and terminally differentiated cells are spatially segregated (7).

PCR analysis of RNA from microdissected populations of lens cells indicates that 10K/MIF expression in lens is strongly associated with cellular differentiation. 10K/MIF is thus a useful marker for the process by which the relatively undifferentiated epithelial cells of the lens undergo terminal differentiation into extremely elongated fiber cells. From a functional perspective, these results also suggest that 10K/MIF may have a general involvement in the differentiation process. Because of the morphology of the lens, expression in the outer epithelium is not inconsistent with a role in communication with the vitreous and the retina. However, this pattern of expression is equally consistent with a direct role in the differentiation of lens cells themselves. Indeed, these two possibilities are not mutually exclusive and there could be real advantages to a mechanism in differentiation that communicated its activity to surrounding tissues for coordination of growth or function.

Lens fiber cells will not grow in culture and are apparently resistant to natural tumorigenesis (24). MRP8 and MRP14 are proteins that share MIF activity and strongly inhibit cell growth in culture (25). Partial sequence analysis reveals that MRP8 or a protein of related sequence is also expressed in lens, suggesting that MIF activity may reflect a related complex of functions in lens and in the immune system. The activity of proteins with such activity may have an important role in the characteristic properties of lens development and perhaps in the differentiation of many tissues.

Whatever the normal role of 10K/MIF in the developing lens, it could have important clinical implications. Certain eye pathologies have been directly associated with circulating MIF activity (26). Leakage of proteins from damaged lenses can trigger uveitis and endophthalmitis, severe ocular inflammatory conditions with macrophage involvement (27, 28). Clearly, lens MIF activity is a good candidate for stimulating any macrophages present in the eye. Indeed, preliminary results suggest that mouse lens extract does have significant MIF activity (V. Paralkar, F. Noonan, and G.J.W., unpublished results).

1. Wistow, G. & Piatigorsky, J. (1988) *Annu. Rev. Biochem.* **57**, 479–504.
2. Berman, E. R. (1991) *Perspectives in Vision Research: Biochemistry of the Eye* (Plenum, New York), 1st Ed.
3. Wistow, G. J., Mulders, J. W. & de Jong, W. W. (1987) *Nature (London)* **326**, 622–624.
4. Piatigorsky, J. & Wistow, G. (1991) *Science* **252**, 1078–1079.
5. Wistow, G. (1993) *Methods Enzymol.*, in press.
6. Weiser, W. Y., Temple, P. A., Witek-Giannotti, J. S., Remold, H. G., Clark, S. C. & David, J. R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7522–7526.
7. Spiewak Rinaudo, J. A. & Zelenka, P. S. (1992) *Exp. Cell Res.* **199**, 147–153.
8. Shaughnessy, M. & Wistow, G. (1992) *Curr. Eye Res.* **11**, 175–181.
9. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989) *Gene* **77**, 51–59.
10. Kanehisa, M. (1988) *IDEAS-88: Integrated Database and Extended Analysis System for Nucleic Acids and Proteins* (Frederick Cancer Research Facility, Frederick, MD).
11. Odink, K., Cerletti, N., Bruggen, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhard, G., Schlegel, R. & Sorg, C. (1987) *Nature (London)* **330**, 80–82.
12. Dorin, J. R., Novak, M., Hill, R. E., Brock, D. J. H., Secher, D. S. & van Heyningen, V. (1987) *Nature (London)* **326**, 614–617.
13. Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J., eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic, New York), 1st Ed.
14. Yamada, T., Nakamura, T., Westphal, H. & Russell, P. (1990) *Curr. Eye Res.* **9**, 31–37.
15. Reddan, J. R., Chepelinsky, A. B., Dziedzic, D. C., Piatigorsky, J. & Goldenberg, E. M. (1986) *Differentiation* **33**, 168–174.
16. Harris, L. H., Talian, J. C. & Zelenka, P. S. (1992) *Development* **115**, 813–820.
17. Weiser, W. Y., Pozzi, L. A. & David, J. R. (1991) *J. Immunol.* **147**, 2006–2011.
18. Walls, G. L. (1967) *The Vertebrate Eye and Its Adaptive Radiation* (Hafner, New York) (facsimile of 1942 Ed.).
19. Cuthbertson, R. A. & Lang, R. A. (1989) *Dev. Biol.* **134**, 119–129.
20. Reddan, J. R. (1982) in *Cell Biology of the Eye*, ed. McDevitt, D. S. (Academic, New York), pp. 299–375.
21. Cuevas, P., Carceller, F., Ortega, S., Zazo, M., Nieto, I. & Gimenez-Gallego, G. (1991) *Science* **254**, 1208–1210.
22. Sato, Y., Shimada, T. & Takaki, R. (1991) *Biochem. Biophys. Res. Commun.* **180**, 1098–1102.
23. Maier, J. A., Voulalas, P., Roeder, D. & Maciag, T. (1990) *Science* **249**, 1570–1574.
24. Mahon, K. A., Chepelinsky, A. B., Khillan, J. S., Overbeek, P. A., Piatigorsky, J. & Westphal, H. (1987) *Science* **235**, 1622–1628.
25. Murao, S. I., Collart, F. & Huberman, E. (1990) *Cell Growth Diff.* **1**, 447–454.
26. Broersma, L., Mourits, M. P., Koornneef, L., Wiersinga, W. M., Prummel, M. P. & Berghout, A. (1989) *Clin. Exp. Immunol.* **75**, 275–279.
27. Nussenblatt, R. B. & Palestine, A. G. (1989) *Uveitis: Fundamentals and Clinical Practice* (Year Book Medical, Chicago).
28. Muller-Hermelink, H. K. & Daus, W. (1986) in *Uveitis. Pathophysiology and Therapy*, eds. Kraus-Mackiw, E. & O'Connor, G. R. (Thieme Medical, New York), 2nd Ed., pp. 155–203.