

Production of Type VI Collagen by Human Macrophages: A New Dimension in Macrophage Functional Heterogeneity^{1,2}

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Macrophages derived from human blood monocytes perform many tasks related to tissue injury and repair. The main effect of macrophages on the extracellular matrix is considered to be destructive in nature, because macrophages secrete metalloproteinases and ingest foreign material as part of the remodeling process that occurs in wound healing and other pathological conditions. However, macrophages also contribute to the extracellular matrix and hence to tissue stabilization both indirectly, by inducing other cells to proliferate and to release matrix components, and directly, by secreting components of the extracellular matrix such as fibronectin and type VIII collagen, as we have recently shown. We now report that monocytes and macrophages express virtually all known collagen and collagen-related mRNAs. Furthermore, macrophages secrete type VI collagen protein abundantly, depending upon their mode of activation, stage of differentiation, and cell density. The primary function of type VI collagen secreted by macrophages appears to be modulation of cell-cell and cell-matrix interactions. We suggest that the production of type VI collagen is a marker for a nondestructive, matrix-conserving macrophage phenotype that could profoundly influence physiological and pathophysiological conditions in vivo. *The Journal of Immunology*, 2008, 180: 5707–5719.

Adult macrophages are the phagocytotic cells of the cellular immune system (1). They are derived from circulating blood monocytes that leave the blood and become resident in tissues. Recruitment of monocytes to peripheral tissues increases when the cells are exposed to proinflammatory stimuli. Inside tissues, monocytes differentiate into macrophages, including splenic and alveolar macrophages, Kupffer cells, Langerhans cells, and osteoclasts, with heterogenic phenotypes and functions. During inflammatory responses, freshly recruited blood monocytes not only differentiate but often undergo a process known as specific activation. The functions of monocyte-derived macrophages depend on the kind of progenitor monocyte, the particular stimulus of activation and the tissue microenvironment within which the cells themselves reside (2, 3). Thus, tissue macrophages and recruited macrophages are heterogeneous with respect to phenotype, morphology, function, and expression of antigenic markers.

Classically activated macrophages arise when blood monocytes migrate into inflamed tissue in response both to proinflammatory cytokines such as IFN- γ and to various activating cues from a perturbed extracellular matrix (ECM)⁴ to neutralize the stimulus of

inflammation (4). These macrophages express proteases that degrade ECM. They also produce additional proinflammatory cytokines that further stimulate the inflammatory process in a positive feedback fashion (1). Thus, classically activated macrophages are generally degradative in nature, destabilizing and destroying ECM, phagocytosing apoptotic cells and debris, and breaking down tissues. In the case of complicated, macrophage-rich atherosclerotic lesions, this process may cause the lesions to rupture, ultimately leading to myocardial infarction and stroke (5).

However, macrophages can also be activated in several other ways depending on the stimulus the cells are exposed to (reviewed in Refs. 2 and 6). For example, the exposure of recruited monocytes to IL-4 or IL-13 generates alternatively activated, anti-inflammatory macrophages, whereas exposure to IL-10 leads to a deactivated, immunosuppressive phenotype. TGF- β 1 also deactivates macrophages to an anti-inflammatory phenotype quite distinct from that of alternatively activated macrophages (7–9).

Different modes of macrophage activation and deactivation are characterized by specific gene expression profiles, which lead to a versatile macrophage functionality (2, 6, 10). Both alternatively activated and deactivated macrophages are characterized by the synthesis of anti-inflammatory cytokines, the induction of ECM synthesis, the stimulation of cell proliferation in neighboring cells, and decreased protease activity (2, 4, 6). Although these macrophages do not elaborate a complex ECM, they do produce some matrix proteins including fibronectin and TGF- β -induced gene H3 (β IG-H3 or TGFBI) (11).

Moreover, upon the phagocytosis of apoptotic cells the phenotype of classically activated macrophages reverts to that of alternatively activated, anti-inflammatory macrophages (2, 4). Alternatively activated macrophages derived from classically activated macrophages contribute to the resolution of inflammation and wound healing (12, 13). Until recently it was thought that the

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⁴Abbreviations used in this paper: ECM, extracellular matrix; β IG-H3, TGF- β -induced gene H3; COL6A, collagen type VI, α -chain; P4HA, prolyl 4-hydroxy-

lase α ; SMC, smooth muscle cell; SRP14, 14 M_r (K) signal recognition particle; T6C, type VI collagen.

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transformation of classically activated macrophages to alternatively activated macrophages is a one-way street (4). However, Watkins et al. have now shown that upon appropriate cytokine treatment (e.g., IL-12) tumor-associated macrophages, which are known to display anti-inflammatory and immunosuppressive features (14), can be reactivated to a proinflammatory state (15).

Duffield and colleagues showed that both classically and alternatively activated macrophages can be present in the same tissue and demonstrated that different macrophage populations play pivotal roles in liver injury and repair (16). The mechanisms that allow different macrophage populations to coexist are unknown, but their coexistence seems to foster a healthy immune response.

Macrophages were long believed not to produce collagens, the major structural proteins of the intact ECM. Recently, we demonstrated that macrophages synthesize and secrete type VIII collagen *in vitro* (17), a known component of certain ECMs. This initial hint that macrophages themselves could contribute to the structural integrity of the matrix by synthesizing collagen was supported by our finding that type VIII collagen synthesis also occurs *in vivo* in macrophages of the atherosclerotic plaque (17).

We reasoned that collagen synthesis in macrophages may be a specific feature of a hitherto unrecognized matrix-conserving macrophage phenotype. In this respect, type VI collagen (T6C) is of particular interest because it forms beaded filaments with a complex multidomain structure that is known to interact with both extracellular matrices and cell surface receptors to anchor interstitial structures and cells into the surrounding tissue (18). T6C is secreted exclusively as an assembled heterotrimer comprised of three different polypeptide chains ($\alpha 1(VI)$, $\alpha 2(VI)$, and $\alpha 3(VI)$) that are encoded by three distinct genes (*COL6A1*, *COL6A2*, and *COL6A3*, where *COL6A* is collagen type VI, α -chain). The 260-kDa $\alpha 3(VI)$ -chain limits molecule assembly (18). Variations in T6C expression and distribution have been reported in many pathological conditions, such as fibrosis, wound healing, myopathies, diabetes, hypertension, alcoholic liver disease, and cancer (18, 19).

We report here that monocytes and macrophages express almost all known collagen and collagen-related mRNAs. We focused our research on T6C due to its special role in tissue stability and because the mRNAs for all three T6C chains were among the most strongly produced mRNAs in our assay. We found that macrophages secrete T6C abundantly depending on their stage of differentiation, mode of activation, and cell density. Interestingly, TGF- $\beta 1$ strongly induced T6C synthesis. TGF- $\beta 1$ therefore evokes a particular macrophage phenotype that is characterized by increased T6C secretion. We now demonstrate that macrophages use T6C to modulate their binding properties rather than to build native ECM as do other cell types, including fibroblasts and smooth muscle cells (SMC). Thus, the discovery of extensive T6C production by activated macrophages adds a new dimension to macrophage functional heterogeneity.

Materials and Methods

Cell culture

Human monocytes were obtained from consenting healthy volunteers by leukapheresis and countercurrent elutriation as previously described (20). The procedure for monocyte isolation was approved by the University Hospital Ethics Committee, Münster, Germany. The purity of the monocytes was checked by flow cytometry and exceeded 95%. Human monocyte-derived primary macrophages were obtained by culturing 10^7 monocytes per 75-cm² flask in RPMI 1640 medium (Sigma-Aldrich) containing 20% autologous human serum (PAA Laboratories), 1% nonessential amino acids, 1% sodium pyruvate, and 0.1 mg/ml penicillin/streptomycin/L-glutamine (Sigma-Aldrich) for 14 days unless stated otherwise. For analyzing T6C production during monocyte-to-macrophage differentiation, the me-

dium was replaced with fresh medium without serum and with 50 μ g/ml ascorbic acid for 24 h before harvesting the cells. The times shown in Fig. 1 denote the days on which the cells were harvested. Ascorbic acid is a cosubstrate for prolyl hydroxylase that catalyzes the posttranslational hydroxylation of proline residues in collagen chains. Its presence in the medium is required for proper triple helix formation in the endoplasmic reticulum and thus for the secretion of procollagen peptides (21). In other experiments, macrophages were stimulated with various cytokines (Merck) at different concentrations as indicated. For these experiments the cells were serum starved in the presence of ascorbic acid and cytokines for 24 h at the concentrations indicated in Figs. 3 and 4.

The THP-1 monocytic cell line was purchased from the American Tissue Culture Collection (ATCC). The cells were maintained in RPMI 1640 containing L-glutamine, 10% FCS (PAA Laboratories), and 0.1 mg/ml penicillin/streptomycin/L-glutamine. THP-1 monocytes were harvested directly from the cell suspension or seeded and differentiated into macrophages using 0.1 mM PMA and 50 nM 2-ME for 4 days unless otherwise stated. Culture conditions for the last 24 h before harvesting were as with human monocyte-derived macrophages.

Human skin fibroblasts from consenting healthy volunteers and the human A7 skin melanoma cell line (ATCC) were cultivated in DMEM (Sigma-Aldrich) containing 10% FCS (PAA Laboratories) and 0.1 mg/ml penicillin/streptomycin/L-glutamine. After reaching 80% confluence, the cells were grown for the last 24 h in DMEM supplemented with 0.1 mg/ml penicillin/streptomycin/L-glutamine and 50 μ g/ml ascorbic acid, but without FCS.

Human coronary artery SMC were purchased from BioWhittaker. Cells were cultured using the SmGM2 (smooth muscle growth medium 2) Bullet kit in medium containing 5% FCS. When 80% confluent, cells were grown for the last 24 h in MCDB medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 4 mM L-glutamine, 100 mM sodium hydrogen carbonate, 30 μ g/ml heparin, 50 μ g/ml ascorbic acid, and 1% FCS (all materials from Sigma-Aldrich).

RT-PCR assays

Total RNA was isolated from cell lysates after shearing of genomic DNA with 1.2×100 -mm TSK Supra Access needles (TSK Laboratories) using the RNeasy mini kit (Qiagen) as previously described (22). After DNase I treatment according to the manufacturer's instructions (Qiagen), cDNA was synthesized by reverse transcription using oligo(dT) primers and RevertAid Moloney murine leukemia virus reverse transcriptase (Fermentas). Polymerase chain reactions were performed using HotStarTaq DNA polymerase (Qiagen) in a Mastercycler epGradientS (Eppendorf). The PCR primers were designed using the Primer Express v2.0 software (Applied Biosystems) as previously described (22). Primers were obtained from Invitrogen Life Technologies and primer sequences are listed in Table I. To avoid PCR amplification of contaminating DNA, the forward and reverse primers of each pair were located in different exons. PCR conditions were as follows: initial heat activation of the Taq polymerase at 95°C for 15 min followed by up to 40 cycles comprised of 95°C for 15 s, 56°C for 30 s, and 72°C for 1 min followed by a final elongation at 72°C for 10 min. Following amplification, PCR products were separated by gel electrophoresis on agarose gels. Complementary DNA segments of collagens with GC contents >70% were amplified in the presence of 0.5 M homoectoine, a new synthetic compatible solute that lowers DNA melting temperature and enhances PCR amplification of GC-rich templates (23).

Real-time RT-PCR

We performed real-time RT-PCR using the ABI PRISM 7900HT sequence detection system (Applied Biosystems) and the QuantiTect SYBR Green PCR kit (Qiagen) as previously described (24). The PCR primers were designed using the Primer Express version 2.0 software (Applied Biosystems) as mentioned above (22). Primers were obtained from Invitrogen Life Technologies and the primer sequences are listed in Table I. Cycling parameters were as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Each reaction contained cDNA corresponding to between 0.2 and 7 ng of total RNA and 200 nM forward and reverse primer (see Table I). For each assay, cDNA samples were measured in duplicate and analyzed using the Sequence Detection System version 2.2.2 software (Applied Biosystems). We performed relative mRNA quantification using the $\Delta\Delta C_t$ threshold cycle method. Expression levels of COL6A1, COL6A2, and COL6A3 were normalized to the expression of housekeeping genes, SRP14 (14 M_r (K) signal recognition particle; NM_003134) or GAPDH (NM_002046). Following PCR amplification, samples were subjected to melting temperature analyses to check purity. The correct sizes of PCR products were confirmed by agarose gel electrophoresis and their identities were verified by sequencing

Table I. RT-PCR primers used in this study^a

mRNA	GenBank Accession No.	Amplicon Size (bp)	Forward Primer	Reverse Primer
COL1A1	NM_000088	61	5'-TACAGCGTCACTGTGCGATGGC-3'	5'-TCAATCACTGTCTTGGCCCCAG-3'
COL1A2	NM_000089	136	5'-CACCCAGAGTGGAGCAGTGG-3'	5'-TTCTTGGCTGGGATGTTTTCA-3'
COL2A1	NM_001844	72	5'-CTGAGACAGCATGACGCCG-3'	5'-GGCTGGGATGCTCTCAAT-3'
COL3A1	NM_000090	130	5'-AATTTGGTGTGGACGTTGGC-3'	5'-TTGTCCGCTCACTTGCCTGG-3'
COL4A1	NM_001845	93	5'-CTACGTGCAAGGCAATGAACG-3'	5'-GCAGAACAGGAAGGCATTGT-3'
COL4A2	NM_001846	108	5'-TGTCTCCATCCCACACTGCC-3'	5'-CCTCTAGACAGCTGCCCGGT-3'
COL4A3	NM_000091	106	5'-CTGGCTGGCTTCATTAACCC-3'	5'-CATGCACACCTGACAGCAGC-3'
COL4A4	NM_000092	95	5'-GAACCTGCCACTTTTTCGAAA-3'	5'-TTAAGGTGTCTGGTCTGGAGC-3'
COL4A5	NM_000495	143	5'-TGGAACTCCTCTGTGCACAT-3'	5'-TGACCGTGGCTCTTTTATTTTC-3'
COL4A6	NM_001847	115	5'-CACTATGCCAGGCCAATG-3'	5'-CACACACAGGACAGCGGT-3'
COL5A1	NM_000093	120	5'-GATTGAGCAGATGAAACGGCC-3'	5'-CCTTGGTTAGGATCGACCCAG-3'
COL5A2	NM_000393	74	5'-GCACGCTTGGCCATATAGA-3'	5'-CCCATTTCACGCCCAAT-3'
COL5A3	NM_015719	77	5'-AATTCAGCTCTTCTCGAGCGG-3'	5'-ACTTTTGGTTCGTCTGGCCAA-3'
COL6A1	NM_001848	146	5'-GACCTCGGACCTGTGGGTAC-3'	5'-TACCCCATCTCCCCCTTAC-3'
COL6A2	NM_001849	113	5'-CTGCGACAAGCCACAGCAG-3'	5'-GGGCACACGATCTGAGGGT-3'
COL6A3	NM_004369	61	5'-GAGCAGCTTGACAACATTGCC-3'	5'-GCCAGAGCACTTGCAGG-3'
COL7A1	NM_000094	136	5'-CATCTTCTCCCTGACGCCTG-3'	5'-CGCACGGTGTGACATTGTCT-3'
COL8A1	NM_001850	146	5'-CGAGCCCGTGATGTACACG-3'	5'-ACTGCCCGGCATACAGTCC-3'
COL8A2	NM_005202	277	5'-GGCAAAGGCCAGTACCTG-3'	5'-CCCCTCGTATTCCTGGCT-3'
COL9A1	NM_001851	83	5'-GCACCGACAGATCAGCACATT-3'	5'-CGCTTAAGACTGGGACCCATC-3'
COL9A2	NM_001852	76	5'-TGCAGGATTTCTGTGTCCAA-3'	5'-ATGCCCTTCACTCCCTGC-3'
COL9A3	NM_001853	87	5'-GATCTGCGACACCTCAGCCT-3'	5'-TTCATGACCGTCCACTGTGC-3'
COL10A1	NM_000493	145	5'-GCCCAGCAGGAGCAAAGG-3'	5'-GGACCGGACTTCTCTGGAT-3'
COL11A1	NM_001854	81	5'-GGTCTGCAGTCGCAATTTTCG-3'	5'-CCACCTAGAGACCCACGGCT-3'
COL11A2	NM_080680	116	5'-TTCCCGATGGAGAGTACTGGG-3'	5'-GACGTCACTCCCTAGGCGTCA-3'
COL12A1	NM_004370	84	5'-CCTGCTAGTGGTGTGTGCA-3'	5'-TCCATATGTGGTCTGTGCTCA-3'
COL13A1	NM_005203	118	5'-CTGAAGCTGCTGCCTCTCCTC-3'	5'-TCTGTTCCGCTGGAAATGTC-3'
COL14A1	NM_021110	102	5'-TCCGAGGAATGGTATAACCCG-3'	5'-TGGACCAGGAACCTGACAGG-3'
COL15A1	NM_001855	107	5'-AGGCCCTGGATGCACAATG-3'	5'-GACCAATTCAGCCGTTGG-3'
COL16A1	NM_001856	84	5'-AGGCAAGGTCTACACCCGCT-3'	5'-TGGCTGATTCTCCCGTCA-3'
COL17A1	NM_000494	87	5'-AGGAAAACACTACGTTACCCGC-3'	5'-AGATGCAAAATTCCTTCGAGG-3'
COL18A1	NM_030582	110	5'-GTGCCACTCGTCAACCTCAA-3'	5'-CCGTCAAAGGAGAAGATGCGT-3'
COL19A1	NM_001858	145	5'-CGGCTGATGCAGTTTCAATTG-3'	5'-CCAGGTCTCCATAAGCTTGG-3'
COL20A1	NM_020882	150	5'-CGTGCTGTGATGATGGTCT-3'	5'-CCGTGTACTGAGTCAAGCC-3'
COL21A1	NM_030820	114	5'-GGGAATTCAGGGGACCCA-3'	5'-AGTCCACGATCACCCTTGTCA-3'
COL22A1	NM_152888	138	5'-ACGCCCTTGTCAACAACCTTCC-3'	5'-GCGTTGTACTCGACTTGTGTG-3'
COL23A1	NM_173465	129	5'-GCTGCTGGCATAAGTGACCC-3'	5'-AAGGCCACAGGTAGCGCAT-3'
COL24A1	NM_152890	150	5'-GGCACACGAGATAAACCAGC-3'	5'-ATGTCTGGCCACCAGCACTG-3'
COL25A1	NM_032518	123	5'-TCGCGAGTAGCATTTGGTTCC-3'	5'-CCTGCATTCAAGTCAAGGCAA-3'
COL26A1	NM_133457	150	5'-CAGCAGCTGAGAGAGGCCCT-3'	5'-GCCACCCCTTTCATCTTGGAG-3'
COL27A1	NM_032888	55	5'-CATCGAGGTTCTCCTGCAACTT-3'	5'-GCTGATGGCAAACCTGACCTT-3'
COL28A1	NM_001037763	121	5'-TGCCTGCCACTACCTCATCTG-3'	5'-TATTCACCACAGTTTCCAGGCTTC-3'
BMP1	NM_006131	51	5'-CTTCTGGCAGCAACACTCG-3'	5'-ACTCCTGCCCTGGCTGGAT-3'
LOX	NM_002317	54	5'-GGACATCATGCGTATGCCTCA-3'	5'-CCACTTCAGAACACCAGGCAC-3'
P4HA1	NM_000917	53	5'-ACCCCATTTTGTGACTTGCACG-3'	5'-CCTGTCCCGACTCTTTGAAA-3'
P4HA2	NM_004199	57	5'-GGCTGCAATTTGGCTAAGAA-3'	5'-TCCGCAAGAGGTTGTACCAGA-3'
P4HA3	NM_182904	51	5'-AGCGTGCCTGTGGTTAGGAAT-3'	5'-TGTGTCACTGTCCCTTACC-3'
P4HB	NM_000918	80	5'-GAGTCTGCCACCGCTTCTT-3'	5'-TGCTGTCCAGTCCCTCCG-3'
PCOLN3	NM_002768	53	5'-CGGAGCAGGCCAAAGTGAA-3'	5'-CACCTTCGTTCTTCTGCGG-3'
BGN	NM_001711	106	5'-GCCAAGCTGACTGGCATCC-3'	5'-AGTAGCGAAGCAGGTCTCTCA-3'
CHAD	NM_001267	166	5'-TCCCAACAACCCCTGAAAAG-3'	5'-GGTCAAGCGGTTGTCTCCA-3'
DCN	NM_001920	101	5'-TGATGCAGCTAGCCTGAAAAG-3'	5'-AGGCGTGTGGCCAGAGAG-3'
FBN1	NM_000138	104	5'-AGCACACTCAGCGGACAG-3'	5'-AAGCCCGCATTACACACGC-3'
FMOD	NM_002023	56	5'-CCCCAGTCAACACCAACC-3'	5'-GTTCAAGCAGCTCCACCAGC-3'
FN1	NM_002026	123	5'-TGATCACATGGACGCTTGC-3'	5'-GAGTCAAGCCGGACACAACG-3'
LUM	NM_002345	149	5'-TCATCCCTGGTTGAGCTGGAT-3'	5'-AGGATAATGGCCCCAGGATCT-3'
MFAP2	NM_017459	52	5'-ACAGTGTGTGCCACAGAGG-3'	5'-CCATCAGCCACATTTGGA-3'
HSPG2	NM_005529	174	5'-TGTGTCGAGATGGAATCAAAGGA-3'	5'-GTCCGACTCTGCTATGCCATGT-3'
SDC1	NM_002997	206	5'-ACAACCTTCCGGCTCAGG-3'	5'-CACTTCTGGCAGGATACAGC-3'
TGFBI	NM_000358	51	5'-TCAAAGATGGAACCCCTCAA-3'	5'-AGTTTTTGGCCGCCAGAGT-3'
VWF	NM_000552	57	5'-GAATGGTGTGTTAGCCTGG-3'	5'-CACGATCGCTCCTGACAC-3'
ITGB1	NM_002211	52	5'-CAAAGCGAAGGCATCCCTG-3'	5'-ACGCCCTTCAATGCACCTG-3'
CSPG4	NM_001897	87	5'-CAGCTGATCCGCTAGTGC-3'	5'-CTGGCGATCCATCTCGGAG-3'
LGALS3BP	NM_005567	59	5'-CTCACTGGCCGACTGCAAG-3'	5'-CTGGTTTCAATGGTGCAGACC-3'
ANTXR1	NM_032208	54	5'-GCCAACGGTAGACGCTTCT-3'	5'-TCTCCCAACGAACCTCCAT-3'
OSM	NM_020530	132	5'-TGCTCAGTCTGGTCTTGCCT-3'	5'-TGCTGGTGTCTGCTGAGATC-3'
SRP14	NM_003134	82	5'-AGCACTGTGGTGTGAGCTCAA-3'	5'-TCAGCCCATCCATGTAGCTCA-3'
GAPDH	NM_002046	115	5'-CAACAGCGACACCCTCTCT-3'	5'-CACCTGTGTGTGAGCCAAA-3'

^a Primers were directed to mRNAs encoding known collagens, proteins known to interact with T6C, proteins involved in collagen processing, or to housekeeping gene mRNAs. Forward and reverse primers are located in different exons.

(BigDye 3.1 terminator cycle sequencing kit; Applied Biosystems). Controls containing all constituents but the template were performed to rule out contamination of reagents.

Metabolic labeling and immunoprecipitation

Mature macrophages were washed three times with serum-, methionine-, and cysteine-free RPMI 1640 medium (ICN Biomedicals) and then incubated with 50 μ Ci/ml Tran³⁵S-Label (ICN Biomedicals) and 50 μ g/ml ascorbic acid in serum-, methionine-, and cysteine-free RPMI 1640 medium. After 24 h the medium was collected, centrifuged, and aliquoted. Immunoprecipitation was performed using a commercial kit (Roche Molecular Biochemicals) as follows. After preclearing using protein G agarose for 1 h, the culture media were incubated with rabbit anti-human T6C Ab (Chemicon) at 4°C in an overhead shaker for 1 h. Protein G agarose was then added and the mixture was incubated overnight at 4°C. After washing according to the manufacturer's instructions, the immunoprecipitate was denatured at 95°C for 5 min in Laemmli sample buffer containing 5% 2-ME and subjected to SDS-PAGE. Radioactive signals were detected using HyperfilmTM x-ray films (Amersham Pharmacia).

Western blot analysis

For analysis of T6C secretion, equal amounts of culture media were separated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore). Tubulin blots of cell lysates were performed to confirm cell viability and the authenticity of the regulation of T6C secretion. After incubation in PBS containing 5% skim-milk for 1 h to block unspecific binding, the membranes were probed with rabbit anti-human T6C Ab (1/5000; Chemicon) or mouse anti- α -tubulin (1/1000; Sigma) for 1 h at 37°C, washed three times in PBS containing 0.5% skim milk and 0.05% Tween 20 for 10 min each, incubated with peroxidase-conjugated secondary Ab (anti-rabbit, 1/100000; anti-mouse, 1/5000; DakoCytomation) for 1 h at 37°C, washed again as described above, and treated with SuperSignal West Femto reagent (PerBioScience) or ECL (Amersham Pharmacia), respectively, in the case of tubulin. Chemiluminescence signals were recorded on Hyperfilm x-ray films (Amersham Pharmacia). Densitometric analyses of Western blots were performed using ImageQuant TL (GE Healthcare).

Confocal immunofluorescence microscopy

THP-1 macrophages were cultured as described above. Mature macrophages were rinsed with PBS and fixed in 4% paraformaldehyde in PBS at room temperature for 30 min. After extensive washing, the cells were incubated for 1 h in PBS containing 1% BSA to block unspecific binding and 0.05% Tween 20 for cell permeabilization, if required. Coimmunolabeling was performed with rabbit anti-T6C Ab (1/200) and mouse anti- β_1 integrin (1/50; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), mouse anti-tubulin (1/1000), or mouse anti-GM130 (1/500; BD Biosciences) Ab, respectively, for 1 h followed by washing and incubation with anti-rabbit Alexa 488- and anti-mouse Alexa 555-conjugated secondary Abs (1/200; Invitrogen Life Technologies). Nuclei were stained with Hoechst 33258 dye. Preparations were mounted in fluorescent mounting medium (DakoCytomation) and examined in an Axiovert 200 M LSM510 confocal laser scanning microscope (Zeiss).

Cell adhesion assay

Wells of 96-well cell culture plates (Biochrom) were coated with 1% BSA or 20 μ g/ml pepsin-solubilized human T6C (Rockland Immunochemicals) in 0.5% acetic acid (Merck) overnight at 4°C. After rinsing with PBS, wells were blocked with 1% BSA in PBS for 1 h at 37°C and washed with PBS. Human THP-1 monocytes were diluted to 10⁶ cells/ml in serum-free RPMI 1640 medium in the absence of PMA or 2-ME. One hundred microliters of this cell suspension was added to each well and cells were allowed to adhere at 37°C for different times. Nonadherent monocytes were removed by gently washing with PBS. Adherent cells were fixed in 1% glutaraldehyde, dried overnight, and stained for 2 h with 1% toluidine blue. After washing with PBS, the chromophore was extracted with 10% acetic acid and absorbance was analyzed at 595 nm on a Tecan GENios ELISA reader.

Electron microscopy

Cultured cells were aspirated from the culture vessels. The remaining ECM was blocked and Ab labeled as described above, fixed in glutaraldehyde, and dried. A goat anti-mouse IgG-12 nm colloidal gold conjugate (Dianova) was used as secondary Ab. Replicas were made by electron beam evaporation of platinum-carbon and carbon at angles of 38° and 90° to thicknesses of 2 and 20 nm. The replicas were incubated overnight in 10% sodium hypochlorite, washed in distilled water, mounted on grids, and

analyzed in a Philips 410LS transmission electron microscope. Surface replicas of cells were obtained by labeling living cells at 4°C as described above.

Statistical analysis

Calculations were performed with Excel version 10.1 (Microsoft) and SPSS version 12.0. Statistical significance was assessed using Student's paired *t* test or one-way ANOVA. In case of real-time RT-PCR data the *p* values were adjusted for multiple comparisons.

Results

Human monocytes and macrophages express collagen mRNAs

Previous work in our laboratory had shown that macrophages synthesize type VIII collagen. We were stimulated by these results to investigate whether macrophages also express other collagens. We therefore designed PCR primer pairs specific for all 42 known collagen chains (Table I) and screened human primary blood monocytes and mature macrophages as well as monocytes and macrophages of the human THP-1 leukemia cell line for human collagen mRNAs. We were surprised to find that these cells expressed almost all known human collagen mRNAs (Table II). In particular, genes encoding chains of types VI, VIII, XI, XVIII, and XXIV collagen were abundantly expressed, whereas genes encoding the chains of types II and III collagen were expressed at very low levels. In fact, only the mRNAs of types XIII and XXII collagen were absent in both monocytes and macrophages of both origins, whereas mRNAs of the remaining collagen chains were expressed at various levels in the different cell types. The mRNAs encoding the α 1(VI), α 2(VI), and α 3(VI) collagen chains of T6C (COL6A1, COL6A2, and COL6A3) were among the most abundantly expressed collagen genes in human macrophages (Fig. 1A). Because T6C is known to stabilize tissues, contribute to wound healing (10), and protect cells against apoptosis (25, 26), this collagen is an excellent marker for macrophages with a matrix-stabilizing phenotype. We therefore focused our further studies on T6C.

Macrophages synthesize T6C de novo

To ascertain whether T6C is synthesized de novo by macrophages, we metabolically labeled and immunoprecipitated secreted T6C in the cell culture medium from mature macrophages. We detected the same banding pattern as in culture media from fibroblasts and coronary artery SMC, which served as positive controls (Fig. 1B). As expected, immunoprecipitation with rabbit IgG yielded no signal. However, the α 3(VI) chain was much more strongly detected than the α 1(VI) and α 2(VI) chains (Fig. 1B), and this discrepancy was also noted in Western blot analyses of cell culture media (Fig. 1C). We suspected this was due to low affinity of the Ab to the α 1(VI) and α 2(VI) chains. We also performed mass spectrometric analyses of immunoprecipitated samples size-separated by SDS-PAGE and confirmed rigorously the identity of the immunoprecipitated α 3(VI) chain (Fig. 2A). Furthermore, after the treatment of macrophage culture media with cathepsin G and neutrophil elastase, proteases known to degrade T6C, secreted T6C could barely be seen in Western blots. Detection was not impaired by incubation with cathepsin L, which does not attack this collagen (Fig. 2B) (27). These data confirm the specificity and efficacy of the anti-type VI collagen Ab for detecting T6C. Clearly, mature macrophages synthesize and secrete T6C.

Expression and secretion of T6C is increased during macrophage maturation

Next, we turned to the questions of when during the differentiation of monocytes to macrophages are the genes for T6C expressed,

Table II. Expression of collagen mRNAs in human monocytes and macrophages^a

mRNA	GenBank Accession No.	Primary Monocytes (4 Days) ^a	Primary Macrophages (14 Days) ^a	THP-1 Monocytes ^a	THP-1 Macrophages (4 Days) ^a	THP-1 Macrophages (6 Days) ^a
COL1A1	NM_000088	●	●	●	●	●
COL1A2	NM_000089	●	●	—	●	●
COL2A1	NM_001844	●	●	●	●	●
COL3A1	NM_000090	●	●	●	—	●
COL4A1	NM_001845	●	●	●	●	●
COL4A2	NM_001846	●	●	●	●	●
COL4A3	NM_000091	—	—	—	●	●
COL4A4	NM_000092	●	●	●	●	●
COL4A5	NM_000495	—	●	●	●	●
COL4A6	NM_001847	●	●	●	●	●
COL5A1	NM_000093	●	●	—	●	●
COL5A2	NM_000393	●	●	●	●	●
COL5A3	NM_015719	●	●	●	●	●
COL6A1	NM_001848	●	●	●	●	●
COL6A2	NM_001849	●	●	●	●	●
COL6A3	NM_004369	●	●	●	●	●
COL7A1	NM_000094	●	●	●	●	●
COL8A1	NM_001850	●	●	●	●	●
COL8A2	NM_005202	●	●	●	●	●
COL9A1	NM_001851	●	●	●	●	●
COL9A2	NM_001852	●	●	●	●	●
COL9A3	NM_001853	●	●	●	●	●
COL10A1	NM_000493	●	●	●	●	●
COL11A1	NM_001854	●	●	●	●	●
COL11A2	NM_080680	●	●	●	●	●
COL12A1	NM_004370	—	—	●	—	—
COL13A1	NM_005203	—	—	—	—	—
COL14A1	NM_021110	—	●	●	●	●
COL15A1	NM_001855	●	●	●	●	●
COL16A1	NM_001856	●	—	●	—	—
COL17A1	NM_000494	●	—	●	—	●
COL18A1	NM_030582	●	●	—	●	●
COL19A1	NM_001858	●	●	●	●	●
COL20A1	NM_020882	●	—	●	●	●
COL21A1	NM_030820	●	●	—	—	—
COL22A1	NM_152888	—	—	—	—	—
COL23A1	NM_173465	●	●	●	●	●
COL24A1	NM_152890	●	●	●	●	●
COL25A1	NM_032518	—	●	—	—	●
COL26A1	NM_133457	●	●	●	●	●
COL27A1	NM_032888	●	●	●	●	●
COL28A1	NM_001037763	—	●	—	●	●

^a ●, Expressed; —, not expressed.

and do only mature macrophages secrete T6C? In primary macrophages the expression of mRNAs for all three T6C chains relative to the expression of the mRNA encoding the housekeeping gene SRP14 increased steadily during differentiation, leveling off after ~7–10 days in culture (Fig. 1D). One-way ANOVA revealed that the decrease in the T6C fibril assembly-limiting COL6A3 mRNA levels from day 7 to days 10 and 14 is not statistically significant. In THP-1 macrophages an initial burst in relative mRNA levels occurring up to day 4 was followed by a noticeable decline by day 6, in particular in the case of COL6A1 and COL6A2 mRNA levels. A slight decrease in COL6A3 mRNA levels from day 4 to day 6 is not statistically significant. To see how macrophage maturation affects T6C secretion, we performed Western blot analyses on the culture medium of monocytes and macrophages at different stages of differentiation (Fig. 1E). We found that monocytes do not secrete T6C at all, while differentiating macrophages and mature macrophages secrete T6C in ever increasing amounts. In primary cells, the observed increase in relative mRNA levels up to day 7 is accompanied by elevated secretion of T6C protein. The decrease in the amount of T6C at day 10 follows the reduced

relative mRNA levels but, interestingly, T6C secretion reaches a maximum at day 14 even though mRNA levels decline (see above). The difference in the amount of secreted T6C between days 7 and 10 is reproducible but, as densitometric and statistical analyses show, not significant ($p = 0.055$; data not shown). We can offer no explanation for the discrepancy between mRNA and protein levels but note that similar phenomena during macrophage maturation have been observed before (28). The reproducible increase in secreted T6C at day 14 following the decline between day 7 and day 10 may be due to an altered state of maturation, because during spontaneous differentiation monocytes usually shift to macrophage-like cells between day 7 and day 10 before they become fully differentiated between days 10 and 14. After this time, macrophages show a seemingly more balanced secretory phenotype.

Ascorbic acid is required for proper secretion of T6C by macrophages

Ascorbic acid is required as a cosubstrate for the posttranslational hydroxylation of proline residues in collagen chains, a process mediated by the enzyme prolyl hydroxylase (21). The

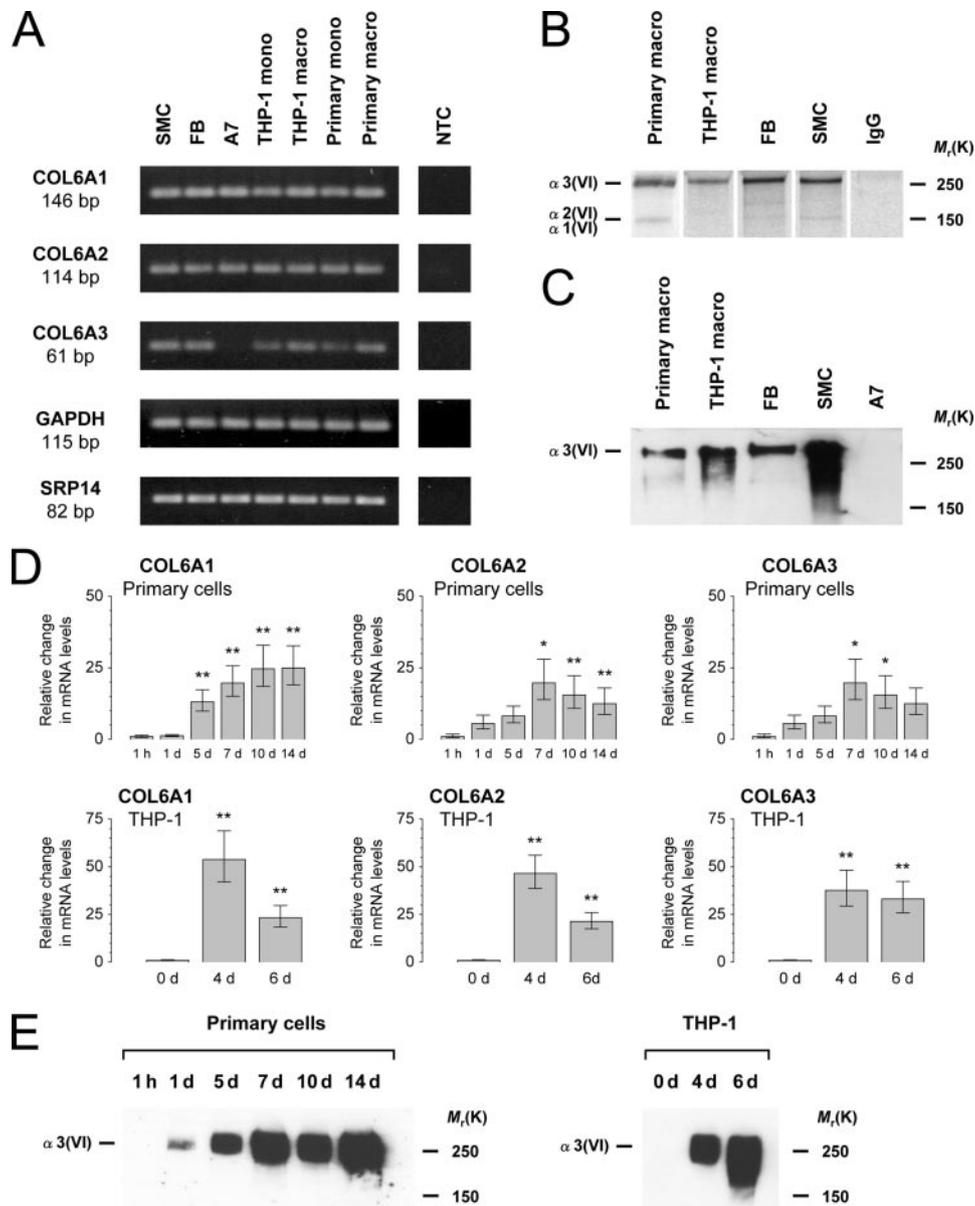


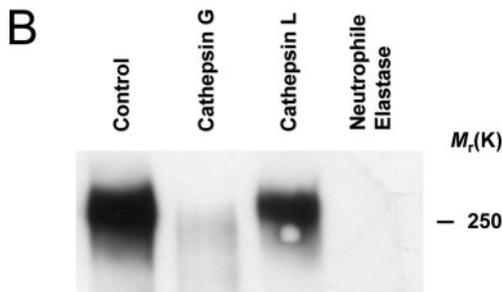
FIGURE 1. Primary and THP-1 monocytes and macrophages express T6C mRNA and macrophages secrete T6C protein. **A**, RT-PCR analysis of T6C mRNAs compared with mRNAs of the housekeeping genes GAPDH and SRP14, a 14 $M_r(K)$ signal recognition particle. Messenger RNA-specific primers located in two different exons of the corresponding mRNAs were used to amplify cDNA fragments derived from T6C mRNAs (COL6A1, COL6A2, and COL6A3). Total RNA was isolated from human cells including coronary artery SMC, skin fibroblasts (FB), A7 skin melanoma cells (A7), THP-1 monocytes (THP-1 mono), 6 day-old THP-1 macrophages (THP-1 macro), and 1-h-old primary monocytes (Primary mono) and 14 day-old primary monocyte-derived macrophages (Primary macro) for RT-PCR. Macrophages express mRNA for all three T6C chains. A7 cells do not express mRNA for the $\alpha 3(VI)$ chain and served as a negative control. NTC, No template controls. **B**, Autoradiographic detection of metabolically labeled, secreted T6C protein. Immunoprecipitation of T6C was performed on culture medium from primary macrophages, THP-1 macrophages, skin fibroblasts, and SMC with a specific Ab to T6C. The control was with rabbit IgG in place of the anti-T6C Ab (IgG). The $\alpha 3(VI)$ chain is strongly labeled, the $\alpha 1(VI)$ and $\alpha 2(VI)$ collagen chains are only weakly labeled. **C**, Western blot analysis of secreted T6C in culture media of primary macrophages, THP-1 macrophages, fibroblasts, SMC, and A7 cells. Fibroblasts and SMC culture media served as positive controls, and A7 cell culture medium served as negative control. Secreted T6C is seen in media from macrophages and the other cells except A7 cells. **D**, Real-time RT-PCR analysis of T6C mRNA expression in total RNA isolated from human primary and THP-1 cells at different times of differentiation relative to the expression of the mRNA encoding the housekeeping gene SRP14. The relative expression of all three T6C mRNAs increased during the differentiation of primary monocytes. The decrease in the T6C fibril assembly-limiting COL6A3 mRNA levels from day (d) 7 to days 10 and 14 is statistically not significant. The relative expression of all three T6C mRNAs in THP-1 macrophages was significantly lower after 6 days of differentiation than after 4 days. However, the decrease in COL6A3 mRNA levels from day 4 to day 6 is not statistically significant. Error bars display the calculated maximum and minimum expression levels representing the SEM expression level ($n \geq 9$). *, $p < 0.05$; **, $p < 0.01$. **E**, Western blot analysis of T6C protein secreted by primary monocytes/macrophages (Primary cells) and THP-1 monocytes/macrophages (THP-1) during differentiation. Monocytes (1 h, primary cells 1 h after isolation; 0 d, THP-1 cells before treatment with PMA) do not secrete collagen, but macrophages do. The amount of secreted $\alpha 3(VI)$ collagen chain (at 260 $M_r(K)$) in the culture medium increases with macrophage differentiation. Although reproducible, the decline in T6C between day 7 and day 10 is not statistically significant.

A

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1 MRKRRHPLVAVFCLFSLGFPPTTHAQQQQADVKNAAAADIIFLVDSWSTIGEEHFQVLFREFLYDVVKSALA
71 VGENDFHFAVLFQFNPNPHTEFLNLTYRTKQEVLSHISNMSYIGGTNGTQKGLLEYIMQSHLTKAAGSRAGD
141 GVPQVIVVLDGDHSGDKGLALPSAELKSDVNVVFAIGVEDADEGALKEIASEPLNMHMFLNENFTSLHDIV
211 GNLVSCVHSSVSPERAGDTETLTKDITAQDSADIIFLIDGSNNTGVSNFVAVILDFLVNLLLEKLPIGTQQIR
281 VGVVQFSDEPRTMFSLDYSTKAQVGLGAVKALGFAGGELANIGLALDFVVENHFFRAGGSRVVEGVPQVL
351 VLISAGPSSDEIRYGVVALKQASVFSFGLGAQAASRAELQHIATDDNLVFTVPEFRSFGDLQEKLLPYIV
421 GVAQRHIVLKPPTIVTQVIEVNRKDIIVFLVDGSSALGLANFNARDFIAKVIQRLEIQDQLIQVAVAQYA
491 DTVRPEFYFNTHPTKREVITAVRKMKPLDGSALYTGSAFDVFRNNLFTSSAGYRAAEGIPKLLVLITGGK
561 SLDEISQPAQELKRSSIMAFATIGNKQADQAELEEIAFDSSLVFI PAEFRAAPLQGMPLGLLAPLRTLSGT
631 PEVHSNKRDIIFLLDGSANVGKTNFPYVRDFVMNLVNSLDIGDNDNIRVGLVQFSPTPVTEfslntyqtks
701 dilgHLRQLQLQGGSGSLNTGSALSYYANHFTEAGGSRIREHVPQLLLLLLTAGQSEDSYLOAANaltrag
771 iltfcvGASQANKAELEQIAFNPSLVYLMDDFSSLPALPQQLIQPLTTYVSGGVEEVLQAPESKRDIIF
841 LFDGSANLVGQFPVVRDFLYKIIDEINLVKPEGTRIAVAQYSDDVKVESRFDEHQSKPEILNLVKRMKIKT
911 GKALNLGYALDYAQRVIFVKSAGSRIEDGVLQFLVLLVAGRSSDRVDGPAENLKGSGVVPFIFQAKNADP
981 AELEQIVLSPAFILAAESLPKIGDLHPQIVNLLKSVHNGAPAPVSGEKDQVVFLLDSEGVRSRSGFPFLKEF
1051 VQRVVEVSLDVGQDRVRVAVVQYSDRTRPEFYLNYSMNKQDVVNAVRLQTLTLLGGPTpntgaalefvlnil
1121 vssAGSRITTEGVPQLLIVLTADRSGDDVRNPSVVVKRGGAAPVIGIGI GNADITEMQTI Sfpidfavaip
1191 frqlGTVQQVISERVTQLTREELSRQLPVLQPLSPVGGKRDVVFLLIDGSSQAGPEFYVVRTLIERLVD
1261 YLDVGDFTTRVAVIQFSDDPKAEFLNNAHSSKDEVDQNAVQRLRPKGRQINVGNALEYVSRNIFKRLPS
1331 RIEEGVPQFLVLISGKSDDEVVVPAVELKQFVAPFTIARNADQELVKISLSPEYVFSVSTFRELPSL
1401 EQKLLTPITTLTSEQIQKLLASTRYPPPAVESDAADivfidsssegvrpdgFAHIRDFVSRIVRRLNIGP
1471 SKVRVGVVQFSNDVFPEFYLYKTYRSQAPVLDAIRRLRGRGSPLNTGKALEFVARNLFVKSAGSRIEDGV
1541 PQHLVLVLGGKSDQDVSRAQVIRSSGIVSLGVGDRNIDRTELQTIITNDPRLVFTVREDELPIEERIM
1611 NSFGPSAATPAPGVDTTPPSREPKKADIVFLDGSINFRRDSFQEVLRVSEIVDTEYEDGDSIQVGL
1681 VQYNSDPTDEFFLKDFTSKRQIIDAINKVVYKGRHANTKVGLEHLRVNHFVPEAGSRDQRPVQIAFVI
1751 TGGKSVEDAQDVSLALTQRGVAVFVAVGVRNIDSEevgkiasnsatafrvGNVQELSELSEQVLETLHDAM
1821 HETLCPGVTDAAKACNLVDVilgfdgsrdqnvfVAQKGFESKvdailnrismhrvscsggrsptvrsvsv
1891 antPSPGVEAFDFdeyqpemlekrfrnmrsqhPYVLTEDTLKVLNKFQRSSPDSVKVVIHFTDgadgla
1961 dlhrasenlrQEGVRALILVGLERVNLERLMHLEFGRGFMYDRPLRLNLLDLDYELAEQLDNI AEAACC
2031 GVPCKCSGQRGDRGPIGSI GPKGI PGEDGYRGYPGDEGGPGERGPPVNGTQGFQGCPCGQGVKGSRGFP
2101 GEKGEVGEIGLDGLDGEDGDKGLPGSSGEGKNPGRRGDKGPRgekgergdvgirgDPGNPGQDSQERGP
2171 getgdlgpmgvPGRDGVGGPGETGKNGGFGRGPPGAKGNKGGPGQPGFEQEGQTRGAQGPAGPAGPPG
2241 LIGEQGISGPRGSGGARGAPGERGRTGPLGRKGEPEGPCKGIGNPGPRGETDGDGRDVGSGEGRGK
2311 GERGFPGYPGPKGNPGEPLNGTTPGKIGIRGRGNSGPPGIVGQKGRPGYPGAPGRNNGSDIDQCALI
2381 QSIKDKCPCCYGPLECPVFPETELAFALDTESEGVNQDTFGRMRDVLSIVNVLTAIESNCTGARVAVVTY
2451 NNEVTTIEIRFADSKRKSVLDDKIKNLQVALTSKQSSLETAMSFVARNTFKRVNRGLMRKVVAVFSSNTPT
2521 RASPQLREAVLKLSDAGITPLFLTRQEDRQLINALQINNTAVGHALVLPAGRDLTDFLNVLTCVCLDI
2591 CNIDPSCGFGSWRPSFRDRAAGSDVIDMAFILDSAEITTLFQFNEMKKYIAYLVRQLDMSDPDKASQ
2661 FARVAVVQHAPSESVDNASMPVKVEFSLTDYGSKEKLVDFLSRGMQLQGLTRALGSAIEYTIENVFESA
2731 PNPRLKIVVLMLTGEVPEQQLEEAQRVILQAKCKGYFFVVLGIRKVNIEKIVTFASEpndvffklvdk
2801 stelnEELPLMFRGLLPSFVSSENAFYLSPIRDKQCDWFQGDQPTKNLVKFGHKQVNVNPNVSSPTSNP
2871 VTTTKPVTTTKPVTTTKPVTTTKPVTTTKPVTTTKPVTTTKPVTTTKPVTTTKPVTTTKPVTTTKPVTTTKPV
2941 AAKPVAAPAAVRPAAAAKPVATKPEVVRPQAAKPAATKPAATKPVVVKMLREVQVFEITENSAKLHWER
3011 PEPGPGYFYDLTIVTSAHDQSLVVKQLNLTVDVIGGLLAGQTYHVAVVVCYLRVSRATYHGSFSTKKSQP
3081 PPPQPARSASSSTINLMVSTELALTEITDICKLPKDEGTCRDFILKWIYDPNTKSCARFWYGGCGGNENK
3151 FGSQKECEKVCAPVLAKPGVISVMGT
    
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FIGURE 2. Mass spectrometry and protease digestion confirm the identity of macrophage-derived secreted T6C protein. **A**, Mass spectrometric analysis of the $\alpha 3(\text{VI})$ chain of T6C secreted by human monocyte-derived macrophages revealed the peptide fragments shown in bold lowercase letters. Overlapping parts of two different fragments are underlined. **B**, Western blot analysis showing that the $\alpha 3(\text{VI})$ chain of T6C in the medium of human monocyte-derived macrophages is degraded by cathepsin G (1 U/ml) and neutrophil elastase (1 U/ml), proteases that specifically degrade T6C, but not by cathepsin L (1 U/ml), which does not attack T6C. Control is without protease.



presence of ascorbic acid is mandatory for proper triple helix formation in the endoplasmic reticulum and for the secretion of procollagen peptides. We therefore investigated the effect of ascorbic acid on T6C secretion by human macrophages. As shown in Fig. 3A, ascorbic acid is required for efficient T6C secretion and dose-dependently increases secreted T6C up to concentrations of 50 μg of ascorbic acid per ml of culture medium. Therefore, 50 $\mu\text{g}/\text{ml}$ ascorbic acid was added to the culture medium in all experiments in which T6C secretion was investigated.

Serum induces secretion of T6C by macrophages

Human autologous serum contains several growth factors, cytokines, and other bioactive compounds. We therefore studied the effect of serum on T6C secretion in the absence of ascorbic acid.

Serum enhanced T6C secretion in mature macrophages in a dose-dependent fashion (Fig. 3B). Western blot analyses revealed that the serum used in our studies contained only minimal amounts of T6C (not shown). Nevertheless, because serum clearly affects T6C secretion, we performed all incubation experiments in the absence of serum.

T6C is actively secreted by macrophages

We next investigated whether macrophages secrete T6C actively via the Golgi apparatus by testing the ability of the protein glycosylation inhibitor tunicamycin and the Golgi blocker monensin to inhibit the secretion of T6C. Dose-dependent depletion of T6C in culture media from mature macrophages occurred after incubating the cells with tunicamycin or GolgiStop, which contains monensin (Fig. 3C). In contrast, secretion of T6C was not

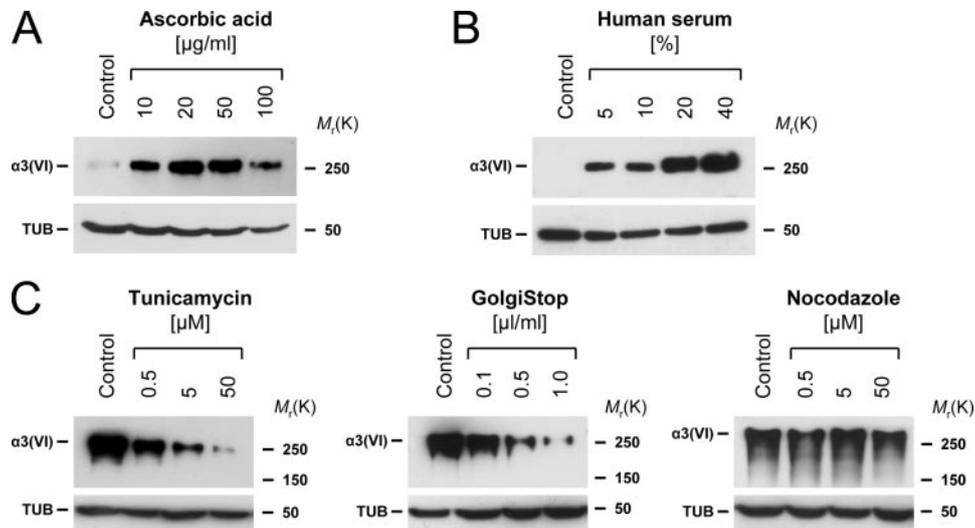
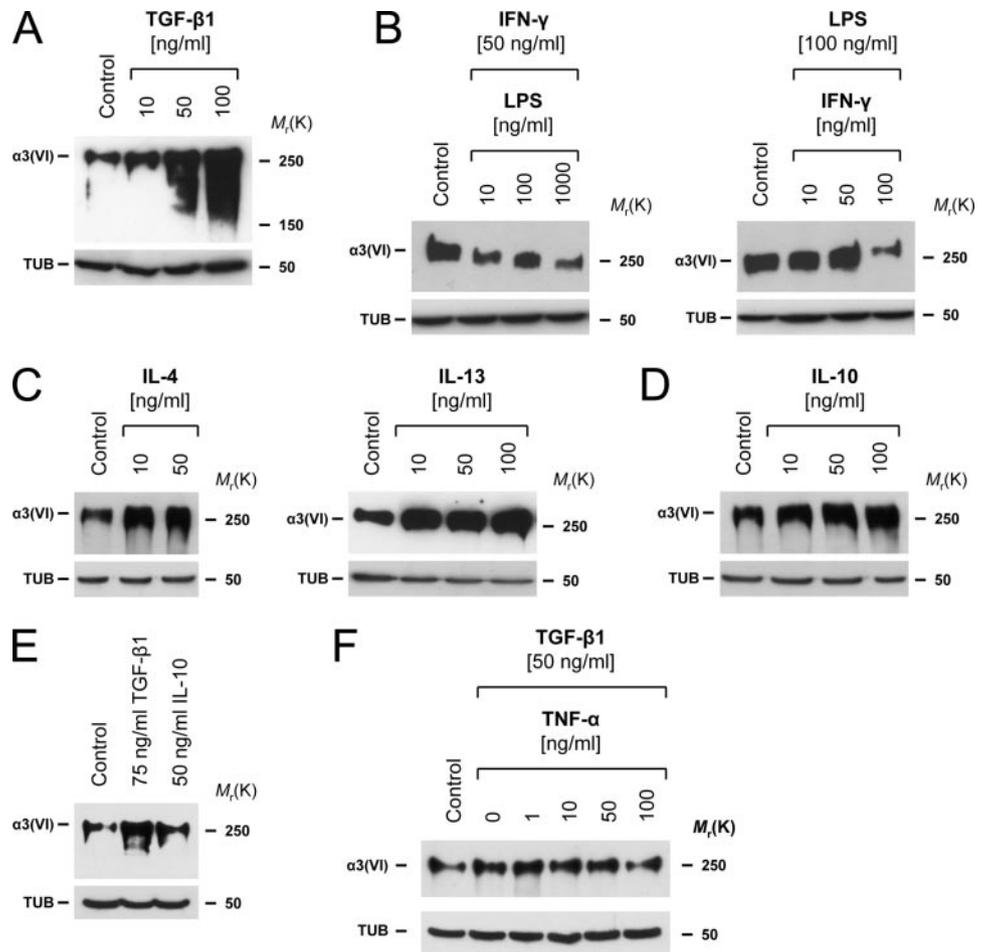


FIGURE 3. Regulation of T6C secretion; Western blot analysis of cell culture media of THP-1 macrophages. *A*, Ascorbic acid, a cosubstrate for the posttranslational prolyl hydroxylase-mediated hydroxylation of proline residues in collagen chains whose presence in the medium is mandatory for triple helix formation in the endoplasmic reticulum and consequently for the secretion of procollagen peptides, is required for efficient secretion of T6C. *B*, Human serum induces secretion of T6C in a dose-dependent manner. *C*, Secretion of T6C after treatment with the glycosylation inhibitor tunicamycin and GolgiStop, which contains the Golgi blocker monensin. The inhibitors reduce the secretion of T6C dose-dependently, indicating that the secretion of T6C depends on glycosylation and that secretion takes place via the Golgi apparatus. Nocodazole, which disrupts microtubules, does not affect T6C secretion, showing that microtubules are not involved. TUB, α -Tubulin.

inhibited by nocodazole, which is known to disrupt microtubules, indicating that microtubules are not involved in intracellular transport or secretion of type VI procollagen or collagen as

reported for type I procollagen (29, 30). Macrophages therefore secrete type VI (pro)collagen via the Golgi apparatus without the involvement of microtubules.

FIGURE 4. T6C secretion by THP-1 macrophages depends on the mode of activation. *A*, TGF- β 1, a potent macrophage deactivator and inducer of ECM production, increases the amount of secreted T6C dose-dependently. *B*, Classical activation induced by a combination of IFN- γ and LPS inhibits the secretion of T6C. *C*, The amount of secreted T6C increases after incubation of the cells with IL-4, and IL-13, both inducers of alternative activation. *D*, IL-10, a deactivator of activated macrophages, increases secreted T6C. α -Tubulin (TUB) is shown to affirm cell viability and the authenticity of regulation of T6C secretion. *E*, At same molar doses TGF- β 1 is a more potent inducer of T6C secretion than IL-10. *F*, The inducing effect of TGF- β 1 is attenuated by TNF- α .



Deactivation by TGF- β 1 increases the amount of secreted T6C

From these and previous results it is clear that macrophages secrete both proteases and collagens. To determine whether macrophages can be shifted toward a collagen-secreting mode, cells were exposed to TGF- β 1, a macrophage deactivator and inducer of ECM synthesis in ECM-producing cells such as fibroblasts and SMC. Western blot analyses of macrophage culture media showed that secretion of T6C was enhanced by TGF- β 1 (Fig. 4A). However, real-time RT-PCR analyses indicated that the induction of T6C secretion is not due to increased T6C mRNA expression (data not shown).

Classical activation decreases whereas alternative activation and IL-10-mediated deactivation increase the amount of secreted T6C

The concept of different modes of macrophage activation proposes different macrophage phenotypes depending on the stimuli the cells are exposed to. We hypothesized that secretion of T6C may be a feature of a novel ECM-stabilizing phenotype. Indeed, the amount of secreted T6C was reduced by the classical activator IFN- γ in combination with LPS (Fig. 4B). In contrast, IL-10, another deactivator of macrophages, as well as IL-4 and IL-13, alternative activators of macrophages (4), increased the amount of T6C in the culture medium (Fig. 4, C and D), but at the same molar amounts not as potently as TGF- β 1 (Fig. 4E). Densitometric and statistical analyses revealed that the enhancement of T6C by all four effectors is significant for all concentrations and that the relative potency of the effectors to induce T6C production is TGF- β 1 > IL-13 > IL-4 and IL-10 (data not shown; $p < 0.05$). The increase in secreted T6C again occurred without affecting the expression of the mRNA of T6C genes (data not shown). The most likely explanation for this discrepancy is that alternatively activated and deactivated macrophages exhibit reduced proteolytic activity (2, 4). However, although TNF- α alone did not affect T6C secretion (data not shown), TNF- α at higher concentrations abrogated the stimulatory effect of TGF- β 1 on T6C secretion (Fig. 4F).

Macrophage T6C is not assembled into beaded filaments but is bound to the cell surface

We viewed immunolabeled T6C secreted by macrophages in surface replicas of cultured cells in the electron microscope. Secreted T6C appeared mainly in peripherally located clusters on the cell surface and is weakly dispersed on the free surfaces of the culture vessels (Fig. 5, A and B). When the macrophages were removed by aspiration to expose the underlying ECM, T6C under the cells was distributed in a similarly disperse fashion (Fig. 5C). Therefore, macrophages clearly did not assemble T6C into beaded filaments as fibroblasts do when treated likewise (Fig. 5D). Western blotting revealed the α 3(VI) chain in extracts of the matrix remaining after the cells were removed, implying that degradation of secreted collagen chains is not the explanation for the lack of beaded T6C filaments in macrophage matrices (Fig. 5E).

Macrophages express proteins required for the processing of collagen peptides

Macrophages may be unable to assemble T6C filaments because they lack the necessary proteins for processing this collagen. We analyzed the expression of mRNAs encoding the collagen-processing proteins prolyl 4-hydroxylase (P4HA1, P4HA2, P4HA3, and P4HB; where A is α and B is β), lysyl oxidase (LOX), type III procollagen N-endopeptidase (PCOLN3) and procollagen C-proteinase (BMP1) in human monocytes and macrophages using real-time RT-PCR (see Table III). Macrophages expressed the mRNAs P4HA1, P4HA2, P4HA3, P4HB, PCOLN3, and BMP1 at levels

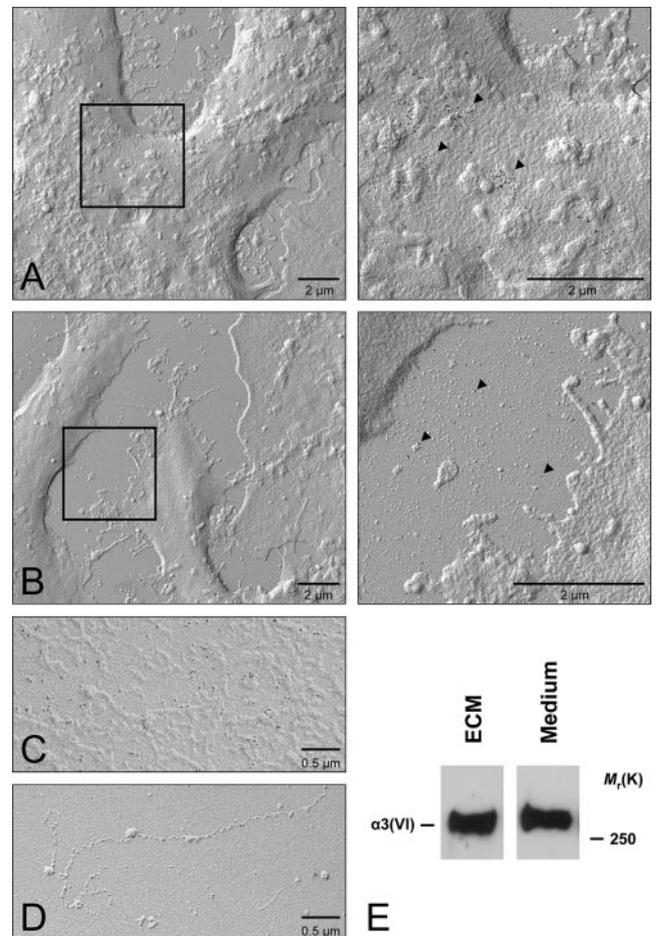


FIGURE 5. T6C secreted by THP-1 macrophages is not assembled into filaments in the ECM. *A*, Surface replicas of macrophages viewed by electron microscopy. Gold-labeled T6C is bound in clusters to the cell surface (enlargement of the boxed area is at *right*). *B*, Scanty labeling of T6C on the culture vessel surface beside the cells (enlargement of the boxed area is at *right*). *C*, Rotary shadowing of matrix underlying macrophages removed by aspiration. *D*, Macrophages do not assemble beaded filaments of T6C as other matrix-producing cells such as fibroblasts do. *E*, Extracts of ECM remaining after removal of macrophages. Extracts contain intact α 3(VI) chains, indicating that at least this chain of T6C is present in the matrix underlying THP-1 macrophages. T6C in the medium of the same culture was a control. Arrowheads in *A* and *B* mark T6C labeling.

comparable to those found in cultured SMC and fibroblasts. In the case of lysyl oxidase, which is important for the cross-linking of several collagens but not T6C (10), expression was much lower than in SMC and fibroblasts. It therefore appears that macrophages are equipped to process type VI collagen correctly.

Proteins required for aggregation, filament assembly, or network formation are expressed at low levels

A second explanation for the absence of beaded filaments of T6C in macrophage matrices may be that macrophages do not synthesize proteins needed to interact with T6C in the matrix. Biglycan (BGN), chondroadherin (CHAD), decorin (DCN), fibrillin (FBN1), fibromodulin (FMOD), fibronectin (FN1), lumican (LUM), microfibril-associated glycoprotein 1 (MFAP2), perlecan (HSPG2), syndecan 1 (SDC1), β IG-H3, and von Willebrand factor (VWF) form a group of ECM proteins that are known to interact with T6C, organizing T6C networks and promoting the aggregation of T6C (31, 32). Using real-time RT-PCR analyses, we screened the expression of the mRNAs encoding these proteins in human monocytes and macrophages (see Table III). Messenger RNAs encoding

Table III. Relative expression of mRNAs encoding various human macrophage proteins involved in collagen processing or that interact with T6C

Protein	miRNA	GenBank Accession No.	Relative Signal Intensity ^a							
			Coronary SMC	Fibroblasts	Primary Monocytes (4 Days)	Primary Macrophages (14 Days)	THP1 Monocytes (1 Day)	THP1 Macrophages (4 Days)	THP1 Macrophages (6 Days)	
Collagen-processing enzymes										
Bone morphogenetic factor 1	BMP1	NM_006131	20.3 ± 0.1	19.7 ± 0.4	20.9 ± 0.6	20.9 ± 0.4	21.2 ± 0.4	20.4 ± 0.1	20.1 ± 0.3	
Lysyl oxidase	LOX	NM_002317	16.5 ± 0.1	17.1 ± 0.2	31.6 ± 0.9	28.6 ± 0.7	24.2 ± 0.3	24.7 ± 0.2	24.9 ± 0.1	
Prolyl 4-hydroxylase α1 subunit	P4HA1	NM_000917	18.1 ± 0.1	18.9 ± 0.1	19.7 ± 0.2	19.6 ± 0.1	18.6 ± 0.1	19.3 ± 0.0	18.8 ± 0.1	
Prolyl 4-hydroxylase α2 subunit	P4HA2	NM_004199	20.3 ± 0.1	19.7 ± 0.1	22.1 ± 0.5	21.6 ± 0.2	24.2 ± 0.2	22.1 ± 0.2	23.4 ± 0.1	
Prolyl 4-hydroxylase α3 subunit	P4HA3	NM_182904	19.8 ± 0.1	22.4 ± 0.1	27.0 ± 0.8	26.5 ± 0.3	27.6 ± 0.2	24.7 ± 0.2	24.4 ± 0.3	
Prolyl 4-hydroxylase β subunit	P4HB	NM_000918	15.1 ± 0.2	15.8 ± 0.6	15.5 ± 0.1	15.7 ± 0.1	14.8 ± 0.0	15.7 ± 0.1	15.4 ± 0.1	
Type III procollagen N-endopeptidase	PCOLN3	NM_002768	18.1 ± 0.6	18.5 ± 1.2	18.3 ± 0.2	18.2 ± 0.2	18.3 ± 0.1	18.2 ± 0.2	18.1 ± 0.2	
ECM proteins interacting with collagen type VI										
Biglycan	BGN	NM_001711	15.3 ± 0.0	17.4 ± 0.1	30.4 ± 1.6	30.5 ± 1.6	30.3 ± 0.3	30.9 ± 0.6	29.8 ± 0.5	
Chondroaderlin	CHAD	NM_001267	32.7 ± 0.8	36.4 ± 4.3			30.6 ± 0.1			
Decorin	DCN	NM_001920	19.2 ± 0.0	14.8 ± 0.0	31.1 ± 0.6	30.2 ± 0.3	28.3 ± 0.6	28.6 ± 0.8	27.2 ± 0.4	
Fibrillin	FBN1	NM_000138	22.8 ± 0.3	22.2 ± 0.4	32.6 ± 0.3	32.4 ± 0.7	30.0 ± 0.4	30.9 ± 1.1	30.8 ± 1.3	
Fibromodulin	FMOD	NM_002023	21.7 ± 0.4	17.8 ± 0.1	30.6 ± 0.7	29.2 ± 0.5	33.1 ± 0.4	32.2 ± 1.1	34.6 ± 2.6	
Fibronectin	FN1	NM_002026	14.4 ± 0.0	18.1 ± 0.2	26.7 ± 1.2	26.7 ± 0.7	26.1 ± 0.2	15.9 ± 0.0	14.9 ± 0.1	
Lumican	LUM	NM_002345	16.5 ± 0.3	13.9 ± 0.0	31.0 ± 2.8	29.0 ± 0.8	25.2 ± 0.3	26.6 ± 0.4	25.2 ± 0.3	
Microfibril-associated glycoprotein 1	MFAP2	NM_017459	18.3 ± 0.2	23.7 ± 0.2	30.1 ± 1.5	29.8 ± 1.0	35.7 ± 2.0	37.6 ± 1.3	37.6 ± 0.5	
Perlecan	HSPG2	NM_005529	17.9 ± 0.1	21.6 ± 0.0	25.0 ± 0.6	23.5 ± 0.7	23.1 ± 0.1	24.2 ± 0.0	22.6 ± 0.1	
Syndecan 1	SDC1	NM_002997	19.9 ± 0.0	19.2 ± 0.1	19.0 ± 0.4	18.8 ± 0.3	22.7 ± 0.2	22.0 ± 0.4	22.6 ± 0.1	
TGF-β-induced gene h3	TGFB1	NM_000358	14.6 ± 0.2	18.0 ± 0.2	18.2 ± 0.2	18.5 ± 0.4	17.1 ± 0.2	17.1 ± 0.2	15.3 ± 0.3	
Von Willebrand Factor	VWF	NM_000552	32.5 ± 0.9	35.9 ± 4.6	28.4 ± 1.9	27.7 ± 1.8		31.7 ± 1.0	32.0 ± 1.1	
Cell surface receptors binding to collagen type VI										
β ₁ integrin	ITGB1	NM_002211	15.5 ± 0.0	14.3 ± 0.0	17.3 ± 0.8	16.6 ± 0.0	17.1 ± 0.2	16.5 ± 0.1	16.0 ± 0.3	
Chondroitin sulphate proteoglycan 4	CSPG4	NM_001897	26.4 ± 0.2	23.9 ± 0.5	24.1 ± 1.5	23.0 ± 1.0	22.3 ± 0.4	21.5 ± 0.2	20.8 ± 0.4	
MAC2-binding protein	LGALS3BP	NM_005567	18.1 ± 0.2	21.1 ± 0.5	21.6 ± 0.2	20.7 ± 0.5	27.3 ± 5.7	20.8 ± 0.3	19.1 ± 0.1	
Tumour endothelial marker 8	ANTXR1	NM_032208	23.0 ± 0.1	21.4 ± 0.2	29.0 ± 1.4	30.8 ± 0.4	25.3 ± 0.4	27.3 ± 0.1	24.6 ± 0.4	
Cytokine binding to collagen type VI										
Oncostatin M	OSM	NM_020530	33.3 ± 1.4	33.9 ± 1.8	25.5 ± 0.7	25.4 ± 0.9	24.1 ± 0.2	22.8 ± 0.1	23.1 ± 0.1	

^a Non-normalized threshold cycle (C_t) values are shown.

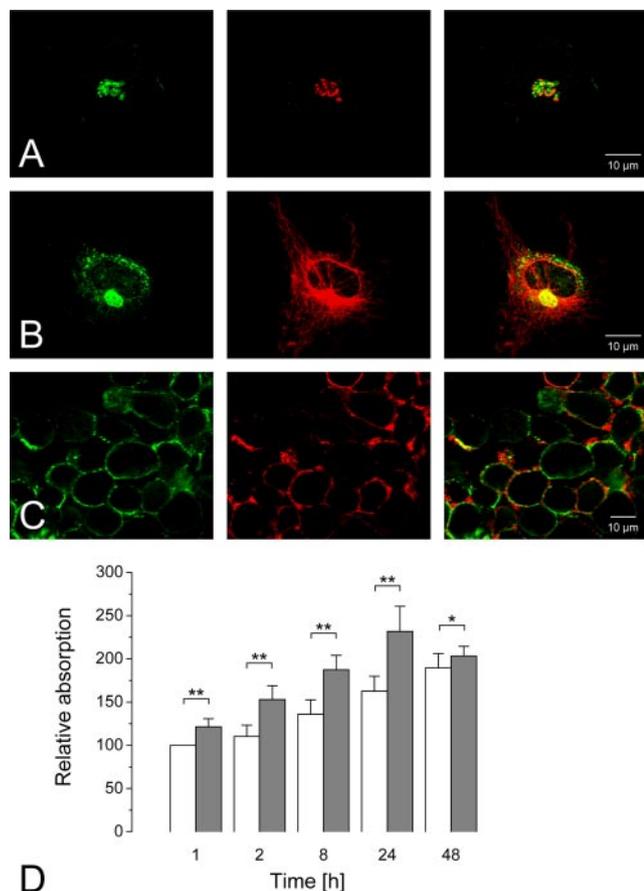


FIGURE 6. Secreted T6C accumulates on the cell surfaces of THP-1 macrophages; T6C enhances adhesion of THP-1 monocytes. *A*, As demonstrated by confocal laser microscopy, macrophages without contacts to adjacent cells contain T6C (green) in the Golgi apparatus (red). Almost no T6C is visible at the cell surface. *B*, T6C (green) is also detected in vesicular structures in the pericellular region but is not colocalized with tubulin (red). *C*, Macrophages seeded at higher cell densities form many cell-cell contacts and accumulate T6C (green) on their surfaces. T6C is partially colocalized with β_1 integrin (red). *D*, Adhesion of THP-1 monocytes to culture vessels coated with BSA and T6C determined photometrically. Adhesion is enhanced when cells are cultured on T6C compared with BSA. Open bars, BSA; gray bars, T6C; *, $p < 0.05$; **, $p < 0.01$.

fibronectin, perlecan, syndecan 1, β IG-H3, and von Willebrand factor were expressed by primary and THP-1 macrophages at levels comparable to those in SMC and fibroblasts. However, in both kinds of macrophages the expression of mRNAs encoding BGN, CHAD, DCN, FBN1, MFAP2, FMOD, and LUM was less than one tenth of that in fibroblasts and SMC. Thus, the absence of T6C fibrils might well be related to the reduced expression of genes encoding specific proteins needed to interact with T6C to promote the elaboration of T6C fibrils.

Macrophages express cell surface receptors that react with T6C

We screened for the expression of mRNAs encoding cell surface receptors known to interact with T6C, including β_1 integrin (ITGB1), chondroitin sulfate proteoglycan 4 (CSPG4), MAC2 binding protein (LGALS3BP), and anthrax receptor 1 (ANTXR1). Real-time RT-PCR analyses revealed that all of these cell surface receptors were expressed at levels comparable to those in SMC and fibroblasts (see Table III). Therefore, macrophages are principally able to bind T6C to their surfaces.

Cell density is an important factor in triggering macrophages to secrete and bind T6C

We investigated the intracellular and extracellular localization of T6C using confocal laser microscopy. Double-staining of THP-1 macrophages with anti-T6C and an Ab to the Golgi marker protein GM130 revealed that a substantial portion of the intracellular T6C was located within the Golgi apparatus (Fig. 6A) and in cytoplasmic vesicles near the nucleus. Using an Ab to tubulin we found no colocalization of the T6C-positive vesicles and peripheral microtubules (Fig. 6B), which is in agreement with the finding above that secretion of T6C is not affected by nocodazole.

Surprisingly, little T6C was detectable at the plasma membrane in THP-1 macrophages cultured at low density (0.4×10^6 cells per 2.55 cm^2 ; Fig. 6, A and B). However, T6C was clearly present at the plasma membrane (Fig. 6C) in cells seeded at high density (0.8×10^6 cells per 2.55 cm^2). At this density the cells were in close contact to each other. T6C was colocalized to some extent with β_1 integrin (Fig. 6C), which is a constituent of several collagen-binding cell surface receptors (33). Vital staining at 4°C of nonpermeabilized macrophages seeded at a high density confirmed that T6C is bound to the cell surface (not shown). These findings provide additional evidence that macrophages do not produce T6C for generating an ECM but rather for promoting the interaction and binding between cells.

Monocyte adhesion is increased on T6C

To determine whether T6C triggers adhesion of monocytes, we performed adhesion assays (Fig. 6D). These showed that initial adhesion of nonstimulated THP-1 monocytes to T6C coatings was much greater than to control coatings of BSA. After 48 h the monocytes adhered in similar numbers to both BSA and T6C. These findings are consistent with the results obtained from the monocyte differentiation series showing that monocytes only begin to secrete T6C after 24 h in culture (Fig. 1E).

Discussion

Macrophages are generally considered to secrete proteases that degrade and destabilize the surrounding ECM (1). However, we previously discovered that macrophages may also promote tissue integrity by producing type VIII collagen (17). We now demonstrate that monocyte-derived macrophages from human blood and cells of the human monocytic THP-1 leukemia line express most known collagen mRNAs, including all three mRNAs of T6C, and that macrophages but not monocytes secrete and capture T6C protein at their cell surfaces.

In our study, the amount of T6C secreted by macrophages was particularly increased by TGF- β 1, a macrophage deactivator and well-known inducer of ECM synthesis in SMC, fibroblasts, and other cells (34). IL-10, another deactivator of inflammatory macrophages, also induced T6C, albeit to a lesser extent than TGF- β 1. However, the interleukins 4 and 13, which induce the alternative macrophage activation pathway (2), also increased the amount of macrophage-derived T6C whereby IL-13 induced T6C more than IL-4 but less than TGF- β 1. Thus, the secretion of T6C appears not simply to characterize a certain mode of macrophage activation. Because alternatively activated and deactivated macrophages both exhibit reduced proteolytic activity in addition to their anti-inflammatory phenotype (2, 4), we suggest that T6C production may even be a marker for an ECM-conserving, nondestructive macrophage phenotype.

Whereas T6C in cells cultured at low density is mainly intracellular, most T6C in cultures at high cell density is captured at the

cell surface where the cells form many cell-cell contacts. In addition, monocytes adhere strongly to adsorbed T6C. Thus, at the cell surface T6C appears to mediate cell-cell interactions and cell adhesion. T6C secreted by macrophages is not present in fibrillar form as is typical of other cells such as SMC and fibroblasts. To gain an explanation for the lack of typical extracellular beaded T6C fibrils, we profiled the expression of genes that encode proteins required for T6C processing and filament formation and genes that encode proteins known to bind T6C (Table III). We found that macrophages express biglycan and decorin, both of which are needed to incorporate T6C into the ECM (31, 32), at very low levels. All of the other genes encoding proteins involved in T6C processing are expressed by macrophages at levels comparable to those of T6C-fibril-producing cells, such as SMC and fibroblasts. Therefore, the virtual absence of biglycan and decorin may explain, at least in part, why macrophages do not incorporate T6C into the ECM in fibrillar form.

Recently, transdifferentiation of monocytes and macrophages into several other cells of the vasculature has been described (35–37). Notably, TGF- β 1 causes macrophages to transdifferentiate into SMC-like cells (38). These studies revealed an increase in SMC markers, including α -actin and calponin, and a decrease in the macrophage marker CD11b after TGF- β 1 treatment for 7 days. Thus, the macrophages in our studies could have conceivably transdifferentiated into SMC, which secrete T6C abundantly. This is, however, unlikely because we treated the macrophages for no longer than 24 h and, although we did not control for expression of SMC markers, the morphology of the cells and the constant expression of the macrophage markers CD68 and 25F9 (data not shown) indicate that the macrophages were not transdifferentiated. Further, whether monocyte transdifferentiation is of real significance in normal homeostasis or tissue repair is controversial; it seems that monocytes contribute to tissue regeneration via cell fusion rather than by transdifferentiation (reviewed in Refs. 39 and 40). In this respect, it remains unclear to what extent the T6C we observed at sites of macrophage-macrophage contact plays a role in the process of macrophage fusion.

Among several possible functions, macrophage-derived T6C may protect against apoptosis as reported for other types of cells (25, 26). However, this also seems unlikely because the cleavage rate of poly-ADP-ribose polymerase in THP-1 macrophages treated with 7-ketocholesterol, a known inducer of apoptosis, was not altered in the presence or absence of anti-inflammatory interleukins that caused enhancement of T6C levels (data not shown). Nevertheless, these results do not preclude a possible role of macrophage T6C in inhibiting apoptosis in adjacent cells.

How macrophages are activated influences their secretory behavior. Vimentin, formerly believed to be exclusively intracellular, has recently been shown to be actively secreted by human macrophages (41). Vimentin secretion is depressed when macrophages are alternatively activated by IL-10 but is elevated upon exposure to TNF- α , indicating that vimentin secretion may be a property of proinflammatory macrophages. However, because T6C mRNA levels are not influenced by these factors, the apparent increase in T6C secretion caused by alternative activators may be a secondary phenomenon resulting from decreased protease activity after activation. Alternative activation and deactivation of macrophages increases the expression of tissue inhibitor of metalloprotease-1 (TIMP-1) and generally suppresses the synthesis of proteases (42–45). Thus, macrophages seem to teeter between proteolytic and matrix-conserving modes depending on external stimuli and the ensuing mode of activation.

The physiological or pathophysiological importance of our finding that human macrophages produce, secrete, and capture T6C is

not immediately obvious. Whether macrophages secrete T6C *in vivo* and, if so, whether the dispersed distribution of T6C in macrophages reflects the distribution in regenerating tissue has to be clarified. However, because T6C is induced by TGF- β 1 and anti-inflammatory interleukins like IL-10 whereas T6C levels are decreased by IFN- γ and LPS, we assume that T6C may be required to attain or maintain the anti-inflammatory, ECM-conserving phenotype of deactivated and of alternatively activated macrophages. This assumption gains further support from the finding that macrophages apparently bind secreted T6C at their surfaces. We assume that T6C together with cell surface receptors containing β 1 integrin might form receptor-ligand pairs similar to the recently described complex of P-selectin and its ligand PSGL-1 (46). The appearance of P-selectin and PSGL-1 (P-selectin glycoprotein ligand-1) on macrophages could potentially be responsible for the formation of aggregates of macrophages and also could cause the binding of macrophages to neutrophils, monocytes, and subsets of T lymphocytes, implicating involvement in the immune response. In a similar manner, macrophage T6C might be a linker molecule that promotes adherence of macrophages and supports macrophage-leukocyte communication and the organization of the immune response. Further studies are needed to test this hypothesis.

Our data and those of other groups indicate that elaboration of the components of the ECM by macrophages may be of paramount importance in tissue remodeling and repair. In addition to type VI and VIII collagen, monocytes and macrophages express and secrete the matrix proteoglycans perlecan and versican (Ref. 47 and Table III). The relevance of this finding is underlined by a study that specifically localized versican to monocytes of infarcted heart tissue (48). Elaboration of versican is accelerated by ischemia/reperfusion and leads to massive cell infiltration and an enhanced inflammatory response. Additionally, the authors showed that treatment with GM-CSF increased the expression of versican. They concluded that up-regulation of versican in the infarcted myocardium influences the inflammatory reaction and mediates subsequent chemotaxis in infarcted heart tissue. Taken together, these and our own findings on collagen secretion substantiate the character of macrophages in injured and/or inflamed tissue. The findings emphasize that to the tasks of macrophages *in vivo* belong not only tissue degradation but also tissue repair and stabilization.

The data presented here imply that the role of macrophages in the remodeling and repair of inflamed or injured tissue is more complex than previously thought. The classic doctrine that macrophages have a primarily proteolytic nature is not valid in general, because appropriately activated macrophages synthesize and secrete several ECM components, including at least two different types of collagens. We envision the production of T6C as a fiducial marker for a nondestructive, matrix-conserving and, hence, probably tissue-stabilizing macrophage phenotype. We believe macrophage-derived collagens may contribute to the structural integrity of tissues and/or modulate cell-cell and cell-matrix interactions when the cells are appropriately activated, especially at higher cell densities as our results show. Our findings may have particular relevance for the atherosclerotic plaque and other tissues in which macrophages are very abundant (17). They may also apply to macrophage-rich tissues in which fibrosis occurs, such as liver and kidney (49), or to macrophage-containing tumors. Additional studies are required to unravel the implications that macrophage-derived collagens harbor for macrophage activity and for tissue integrity.

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Disclosures

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