AS02 - a monoclonal antibody for the detection and elimination of human fibroblasts in vitro

Clone:	AS02
Host:	mouse
Isotype:	IgG1
Specificity:	human fibroblasts
	nerve cells
	few CD34+ blood stem cells
Antigen:	CD90/Thy-1

Immunohistological findings using the monoclonal antibody AS02

Binding of the AS02 on human skin section;(magnification x 150), detected by alkaline phosphatase-anti-alkaline phosphatase (APAAP) and New Fuchsin/naphthol biphosphate (DAKO) (positive cells stain red)

The antibody stained only fibroblasts of the connective tissue of the skin and of the perivascular connective tissue.

Binding of AS02 on fibroblasts of the sebaceous gland basal lamina in a skin section; (magnification x 300), detected by alkaline phosphatase-anti-alkaline phosphatase (APAAP) and New Fuchsin/naphthol biphosphate (DAKO) (positive cells stain red)

Double labeling of a skin capillary with biotinylated AS02 (brown staining with streptavidin-peroxidase and diaminobenzidine/H2O2) and anti-CD31 (endothelial cell marker PECAM-1
(red staining with APAAP and New Fuchsin/naphthol biphosphate); (magnification x 300).

There are no double-labeled endothelial cells in the section.

Binding of AS02 to dermal connective tissue fibroblasts and the perivascular basal lamina.
Note that there is no binding of AS02 to the inner endothelial cell layer of the longitudinally sectioned capillary (C) and of the keratinocytes of the transversely sectioned hair follicle (HF), (magnification x 300), APAAP and New Fuchsin/naphthol biphosphate (red staining of positive cells)

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Double labeling of dermal sweat glands with AS02 (green fluorescence with FITC-labeled second antibody) and anti-smooth muscle actin (SM-actin; red fluorescence with goat anti-mouse CY3 conjugate); (magnification x 600)

Double labeling of dermal smooth muscle cells with AS02 (green fluorescence with FITC-conjugated second antibody) and SM- actin (red fluorescence with goat anti-mouse CY3 conjugate); (magnification x 600)

The figure clearly shows that smooth muscle cells are stained only by the anti-SM-actin antibody. The green stained area between the muscle fibers represents fibroblasts of the connective tissue septa.

Double labeling of a human skin section with anti-CD68 as macrophage marker (red fluorescence with goat anti-mouse CY3 conjugate) and AS02 (green fluorescence with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF)-conjugated goat anti-mouse second antibody), (magnification x 600)

Clearly, AS02 and CD68 antibodies stain distinct cell populations, and no double- labeled cells are observed. These results indicate that tissue macrophages are not recognized by AS02.

Double labeling of dermal Langerhans cells (LC) with AS02 (green fluorescence with DTAF-conjugated goat anti-mouse second antibody) and anti-CD1a (red fluorescence with secondary goat anti.mouse CY3 conjugate). (magnification x 600).

The Langerhans cell-specific anti-CD1a stains only Langerhans cells. There is no detectable double labeling with AS02.

Distribution of AS02 binding in dense connective tissue (tendon); (red staining with APAAP and New Fuchsin/naphthol biphosphate. (magnification x 300)

Fibroblasts of the tendon (T) and of the peri- and endomysium (M) are clearly labeled. Individual fibroblasts of the connective tissue septa between the muscle fibers are stained red with AS02.

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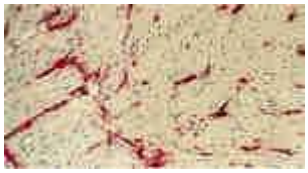
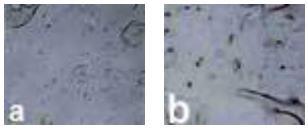


Distribution of AS02 staining on smooth muscle (stomach wall), red staining with APAAP and New Fuchsin/naphthol biphosphate. (magnification x 300).

Only fibroblasts in the connective tissue septa are stained. The smooth muscle cells are not recognized by
AS02.



Binding of AS02 (a) and anti-vimentin (b) in human cartilage; red-brown staining with APAAP and New Fuchsin/naphthol biphosphate. (magnification x 150)

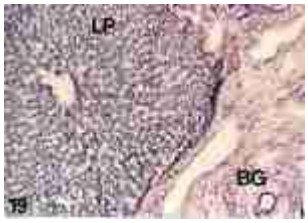
The antibody AS02 does not label mesenchymal chondrocytes. In contrast, these cells are clearly red-brown labeled with the anti-vimetin antibody.

Binding of AS02 to a lymph node section; red staining with APAAP and New Fuchsin/naphthol biphosphate. (magnification x 300).

Marginal zone lymphozytes (MZ) and those of the germinal center (KZ) are not labeled with AS02. Fibroblasts of the connective tissue capsule, the matrix of the lymphoid follicle, and the reticular connective tissue are clearly labeled.

Distribution of AS02 binding in a thyroid follicle section; red staining with APAAP and New Fuchsin/naphthol biphosphate. (magnification x 600)

The follicular monolayer of the thyroid follicle (SF) is not stained by AS02. Only the fibroblasts of the perifollicular connective tissue are detected by this antibody.



Distribution of AS02 staining in a human liver section (19) and in a section of gall bladder wall (20); red staining with APAAP and New Fuchsin/naphthol biphosphate. LP liver parenchyma, BG liver connective tissue, Ep epithelium; (magnification x 150 [liver] and x 300 [gall bladder]).

AS02 does not react with liver parenchymal cells (LP). Essentially, only the fibroblasts of the liver connective tissue (BG) and those of the perivascular connective tissue are stained with AS02.
Gall bladder wall epithelium (Ep) and the smooth muscle cells underlying the lamina propria are not stained. Connective tissue fibroblasts in the lamina propria and in the loose connective tissue between the muscle bundles are clearly stained with AS02.

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Distribution of AS02 staining in a human kidney section; red staining with APAAP and New Fuchsin/naphthol biphosphate, G: glomerulus, pT: proximal tubule, T: tubule. (magnification x 150)

In the kidney, AS02 stains fibroblasts of the tight connective tissue of the capsule and of the reticular connective tissue. Kidney parenchyma is essentially not stained. In a few glomeruli (G), a small number of AS02-positive cells are observed. In most proximal tubules (pT), binding of AS02 to the luminal side is visible.

Distribution of AS02 binding in a human placenta section; brown staining with peroxidase-conjugated goat anti-mouse antibody and diaminobenzidine/H2O2. Ep: epithelium, End: endothelium, M: mesenchyme. (magnification x 300).

AS02 intensely stains all mesenchymal fibroblasts. The endothelial cells of the sectioned lumen are clearly not recognized by AS02. All epithelial structures such as amnion, cytotrophoblast, and syncytiotrophoblast are likewise not stained by this antibody.

Summary of the immunohistological findings using the monoclonal antibody AS02

tissue	cells	monoclonal antibody AS02
skin	fibroblasts	positive
	keratinocytes	negative
	endothelial cells	negative
	smooth muscle cells	negative
	gland cells	negative
	macrophages	negative
	Langerhans cells	negative
cartilage	chondrocytes	negative
muscle	smooth muscle cells	negative
	heart muscle cells	negative
	skeletal muscle cells	negative
lymph node	fibroblasts	positive
	lymphocytes	negative
thyreoid / liver / gall bladder / kidney	fibroblasts	positive
	epithelial cells	negative
	thyroid cells	negative
	liver parenchymal cells	negative
	kidney parenchymal cells	negative
	kidney tubule cells	few positive
placenta	mesenchymal fibroblasts	positive

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	endothelial cells	negative
	epithelial cells	negative
brain	nerve cells	positive

Using flow cytometry with the monoclonal antibody AS02

Indirect immunofluorescence with AS02 and anti-prolylhydroxylase antibody on cultured human dermal fibroblasts; staining with AS02 followed by goat anti-mouse IgG DTAF conjugate (green fluorescence) and the anti-prolylhydroxylase antibody with goat anti-mouse CY3 conjugate (yellow fluorescence). (magnification x 300)

AS02 homogenously stains the entire cell surface, including the numerous pseudopods. A clustering or intensification of staining at cell-cell contact sites was not demonstrated.

Confocal laser scanning microscopic analysis shows a sharp AS02 labeling of the cell membrane and a diffuse cytoplasmatic staining, suggesting that the antigen occurs predominantly at the cell surface, but also in the cytoplasm.

Immunoelectronmicroscopic analysis with pre-embedding labeling also indicates a homogenous distribution of the antigen recognized by AS02 over the entire cell membrane. By these methods, AS02 is shown not to bind to pericytes, endothelial cells, or to components of the extracellular matrix. In the case of pericytes, a false negative result cannot be excluded, given that the basal membrane surrounding these cells is likely to preclude efficient penetration of the antibody. In addition, the post-embedding labeling method allows the detection of a clustered antigen distribution in the fibroblast cytoplasm.

AS02-affinity chromatography has allowed the isolation of an antigen extracted from membranes of cultured fibroblasts[2]. The purified protein has been biochemically characterized (molecular size, primary structure, glycosylation, and membrane attachment via C-terminal glycophosphatidylinositol [GPI] anchor as human Thy-1/CD90, a member of the immunoglobulin superfamily. REIF und ALLEN[3], Dalchau und Fabre[4] und GORDON et al.[5] have shown that Thy-1 is mainly expressed on nerve cells in the human (in distinct contrast to the abundant and eponymous expression on thymocytes in mice and rats), but also other distinct tissues such as kidney. Our results with AS02 on kidney sections confirm these findings.

Immunoblot analysis of detergent extracts of fibroblast membranes reveals a 30-35 kDa protein under reducing and nonreducing conditions, with better reactivity using nonreduced sample. The epitope bound by AS02 resides within the 15-kDa polypeptide chain, and not in the N-linked oligosaccharide side chains or the GPI anchor structure.

Summary of the results using flow cytometry with the monoclonal antibody AS02

cells	reactivity AS02
human fibroblasts	positive
skin	positive
heart	positive
lung	positive

synovia	positive
human keratinocytes	negative
human endothelial cells (EC) microvascular EC (HDMEC)* macrovascular EC (HUVEC)*	 negative negative
human chondrocytes	negative
human blood cells lymphocytes monocytes granulocytes thrombocytes	 negative negative negative negative
$CD34^+$ blood stem cells	20% positive

*HDMEC: human dermal microvascular endothelial cells
**HUVEC: human macrovascular endothelial cells from umbilical vein

Results of the species specificity of the AS02

species	material	AS02	method
human	cultured skin fibroblasts	positive	Flow cytometry
		positive	Immunhistology
rat	cultured fibroblasts	negative	Flow cytometry
mouse	cell line of fibrosarcoma L929	negative	Flow cytometry
	skin	negative	Immunohistology
rabbit	skin	negative	Immunohistology
pig	skin	negative	Immunohistology

Reactivity of AS02 with human endothelial cells

In sections of healthy tissue (skin, placenta, liver, heart), AS02 does not react with endothelial cells. Flow cytometric analysis of cultured micro- and macrovascular endothelial cells similarly shows no binding of AS02.

However, when first activated by phorbol myristate acetate (PMA), tumor necrosis factor-α (TNF-α), or interferon-γ (IFN-γ), in some cases and to differing extents the binding of AS02 to so-called activated endothelial cells could be demonstrated. These studies conducted in vitro have been confirmed by observations made in vivo on pathological tissues associated with various inflammatory diseases.

For example, endothelial cells of rheumatic synovia are AS02-positive, endothelial cells of Kaposi´s sarcoma are stained, and endothelial cells associated with chronic vasculitis react with AS02. It may be concluded that resting endothelial cells are AS02-negative, and that activated endothelial cells are capable expressing Thy-1 and binding AS02. Thus, the antigen recognized by AS02, Thy-1/CD90, can be considered to be an activation marker of human endothelial cells, the physiological function of which (like that of Thy-1 in general), is unknown.

Immunoblot analysis of the AS02

Immunoblot analysis of the AS02 Partial purification of CD90 (Thy-1) from human fibroblasts The monoclonal antibody AS02 was initially described to react specifically at the surface of human fibroblasts, and has recently been shown to react with human Thy-1 antigen (CD90; Saalbach et al. 1998, Arch. Dermatol. Res. 290, 360-366). While biochemically pure Thy-1 is prerequisite for the development of a quantitative enzyme-linked immunosorbent assay (ELISA), partially purified material is adequate for qualitative immunoblot analysis. To this end, 2x106 human primary fibroblasts were harvested by scraping or trypsin/EDTA treatment and pelleted in a microcentrifuge tube. The pelleted cells were extracted with chloroform/methanol (2:1 v/v; modified from the method described for the purification of rat brain Thy-1 by Campbell et al. 1981, Biochem. J. 195, 15-30) at room temperature, and the resulting protein pellet was dried under vaccuum. The dried protein pellet was then extracted in 0,3 ml 1% w/v Triton X-114 in 20 mM Tris-HCl pH 7,5 150 mM NaCl (TBS; see Etges et al. 1986, EMBO J. 3, 597-601) on ice for 10 min. Detergent-insoluble material was pelleted for 1 min at 10'000 x g at 4°C. The supernatant was incubated for 5 min at 37°C , then centrifuged at room temperature for 1 min at 10'000 x g. The upper aqueous phase was discarded, and the lower detergent-enriched phase containing Thy-1 (and some other amphiphilic cellular proteins) was diluted to 0,5 ml with TBS.

Immunoblot analysis was carried out under the conditions described below. goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma) in1:10'000 dilution; development with nitroblue tetrazolium and 5-bromo 4-chloro indolylphosphate in 10% diethanolamine pH 9,2 plus 1 mM MgCl2 An aliquot of 50 µl of the Triton X-114 extract was diluted to 250 µl with distilled water. Laemmli sample buffer without reducing agent (100 µl of a 4x stock solution) was added and the sample heated at 100°C for 3 min. The sample was cooled on ice prior to application to a 0,75 x 6 x 8 cm 4-16% polyacrylamide gradient gel containing SDS. After electrophoresis, the separated proteins were electrophoretically transferred to a sheet of polyvinylidene difluoride (PVDF) using standard methods. The blot was saturated in TBS containing 0,2% Tween 20 (TBST) and 2% bovine serum albumin for at least 30 min at room temperature. The saturated blot was cut into 3-4-mm-wide strips and incubated with control AS02 (0,6 µg in 1 ml TBST-BSA) overnight at room temperature with constant shaking. The strips were washed three times with TBST, then incubated with goat anti-mouse IgG-alkaline phosphatase conjugate (1:10'000 dilution in TBST-BSA). The immunoblot strips were developed with nitroblue tetrazolium and 5-bromo 4-chloro indolylphosphate in 10% w/v diethanolamine pH 9,2 containing 1 mM MgCl2. The AS02 antibody reacting with Thy-1 is revealed as a diffuse blue band at 30-32 kDa.

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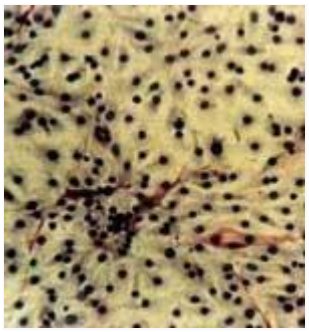
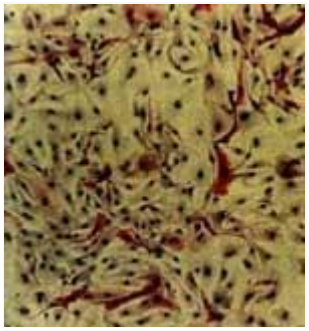
Elimination of fibroblasts from primary cell cultures

Contamination with fibroblasts is common in primary cultures of various cell types. Fast-growing fibroblasts can rapidly overgrow the desire cells in vitro, making it difficult to draw any meaningful conclusions from the resulting mixed cultures. Thus, a rapid and efficient means to eliminate fibroblasts is essential for reproducible results with primary cell cultures. AS02 coupled to magnetic beads allows an efficient means to remove fibroblasts from mixed cultures. Clearly, the same method allows the isolation of small numbers of fibroblasts from such cultures for further analysis.

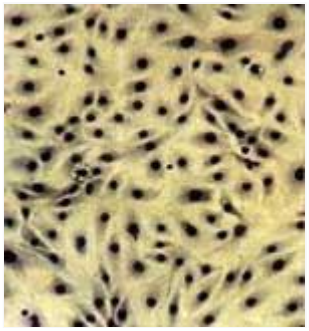
Elimination of contaminating fibroblasts from thyrocyte cultures; the fibroblasts appear red after AS02 binding with the nuclei counterstained with haemalaun.



Thyrocyte culture after the first passage; single fibroblasts (red) are visible

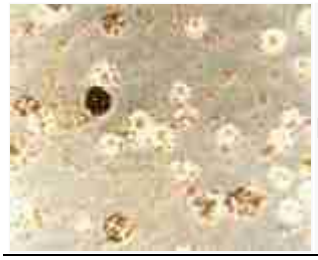


Thyrocyte culture after the fourth passage; many fibroblasts are visible



Thyrocyte culture after elimination of fibroblasts using AS02 magnetic beads (fifth passage)

Elimination of contaminating fibroblasts from microvascular endothelial cell culture using AS02 coupled to magnetic beads



Binding of AS02-microbeads to fibroblast contamination

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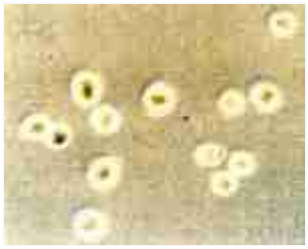

Non-bound endothelial cells immediately after magnetic bead separation of the fibroblasts

References

Saalbach A, Haustein UF, Anderegg U. A ligand of human thy-1 is localized on polymorphonuclear leukocytes and monocytes and mediates the binding to activated thy-1-positive microvascular endothelial cells and fibroblasts. J Invest Dermatol 2000 Nov;115(5):882-8

Saalbach A, Wetzig T, Haustein UF, Anderegg U. Detection of human soluble Thy-1 in serum by ELISA. Fibroblasts and activated endothelial cells are a possible source of soluble Thy-1 in serum. Cell Tissue Res 1999 Nov;298(2):307-1

Saalbach A, Kraft R, Herrmann K, Haustein UF, Anderegg U. The monoclonal antibody AS02 recognizes a protein on human fibroblasts being highly homologous to Thy-1. Arch Dermatol Res 1998 Jul;290(7):360-6

Saalbach A, Aust G, Haustein UF, Herrmann K, Anderegg U. The fibroblast-specific MAb AS02: a novel tool for detection and elimination of human fibroblasts. Cell Tissue Res 1997 Dec;290(3):593-9

Saalbach A, Anderegg U, Bruns M, Schnabel E, Herrmann K, Haustein UF. Novel fibroblast-specific monoclonal antibodies: properties and specificities. J Invest Dermatol 1996 Jun;106(6):1314-9