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DOI
10.1002/(SICI)1097-0029(19970515)37:4<285::AID-JEMT4>3.0.CO;2-K

Publication date
1997

Published in
Microscopy Research and Technique

MICROSCOPY RESEARCH AND TECHNIQUE 37:285–298 (1997)

The Contribution of Quantitative Confocal Laser Scanning Microscopy in Cartilage Research: Chondrocyte Insulin-like Growth Factor-1 Receptors in Health and Pathology

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KEY WORDS arthritis; chondrocyte; in situ; CLSM; quantification

ABSTRACT The use of immunohistochemical detection techniques and fluorescent molecular probes in light and fluorescence microscopy allows accurate and specific analysis of a great variety of cell and tissue components. However, when staining yields only low intensity levels, serious problems may arise with discrimination of specific signals against background staining. This problem is often inherent with articular cartilage research. Application of confocal laser scanning microscopy (CLSM) can circumvent these problems. The CLSM collects images that are almost free of out-of-focus signals, which results in improved spatial resolution and discrimination as compared with conventional microscopy. Moreover, CLSM allows optical sectioning of specimens and three-dimensional reconstruction of the microscopical object. Quantitative evaluation of microscopical images is hampered by out-of-focus signals because they interfere with specific signals in the image. Interference of these nonspecific signals can be diminished by application of CLSM; in CLSM only one single point in microscopical objects is illuminated at any time and this point is then imaged into the pinhole at the entrance of the photo-detector and subsequently digitized. The present review is a discussion of the present state of the art in digital imaging with the use of CLSM in cartilage research. This discussion includes aspects such as sensitivity, specificity, spatial resolution and accuracy of quantitative analysis in microscopical immunofluorescent objects. *Microsc. Res. Tech. 37:285–298, 1997.*

CLSM IN ARTICULAR CARTILAGE RESEARCH

In the field of in situ cartilage research only a few reports are available describing the use of CLSM. Some recent studies will be highlighted here demonstrating the advantage of CLSM over conventional microscopy to localize and quantify immunofluorescence in cartilage tissue.

Improved spatial resolution of immunofluorescent signals in cryostat sections of cartilage, cultured cartilage slices or isolated cells has been obtained with the use of CLSM. This advantage enables collection of detailed information about the in situ localization of matrix molecules (White et al., 1987). For instance Wotton et al. (1991) demonstrated that type IX collagen is localized preferentially in a very small area around individual chondrocytes in cryostat sections of chondrons. Moreover, type VI collagen distribution patterns were analyzed with CLSM by Poole et al. (1992) in isolated chondrons embedded in agarose monolayers. Type VI collagen was found in the pericellular matrix around chondrocytes. Staining was lower in the tail region of chondrons and in the interconnecting segments between adjacent chondrons, whereas lowest amounts of staining were observed in territorial and interterritorial matrices. On the basis of these observations, a dual role of type VI collagen in the maintenance of chondron integrity was suggested. By binding of the radial collagen network it stabilizes collagens, proteoglycans and glycoproteins of the pericellular microenvironment. Furthermore, it provides anchorage and signaling between the pericellular matrix and the nucleus of chondrocytes by mediating interactions via specific cell surface receptors on chondrocytes. CLSM analysis of fixed and stained preparations of isolated chondrocytes revealed heterogeneous intracellular distribution patterns of cathepsin B immunofluorescence in relation to differentiation of chondrocytes (Wotton et al., 1991).

Metabolic events can also be directly monitored with CLSM in living cells, cultured tissue or slices of vital tissue. Metabolic studies in living biological material will become increasingly important in the coming years (Gualtieri, 1992; Menger and Lehr, 1993; Van Noorden and Jonges, 1994). Wuthier et al. (1993) measured Ca^{2+}-fluxes over the cell membrane of chondrocytes in slices of living cartilage by using a fluorescent Ca^{2+}-probe and digital images. In that study, the application of CLSM provided the opportunity to link calcification processes at the cell membrane with exact localization of certain metabolic processes. Guilac (1994) reported

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Received 6 October 1994; Accepted 13 December 1994

morphometric data of living chondrocytes in situ as obtained with CLSM.

The possibility of making optical sections through the depth of specimens is another important advantage of CLSM (Lichtman, 1994; Wilson, 1989). Hirsch and Svoboda (1993) demonstrated that evaluation of optical sections obtained by CLSM of intact tissue samples after immunohistochemistry or in situ hybridization gave similar results as the analysis of conventionally stained tissue sections. Localization of mRNA of F-actin as part of the cytoskeleton as well as mRNA of type II collagen and collagen-containing endoplasmatic reticulum could be demonstrated in optical sections of intact chicken embryonic sterna. This indicates that CLSM optical sectioning can replace conventional sectioning with the advantage of keeping cell and tissue architecture intact.

Although CLSM implies the digitization of a well-defined volume (Mossberg et al., 1990; Van Oostvelt and Bauwens, 1990; Wells et al., 1989), surprisingly few attempts have been made to obtain quantitative information from digital images in cartilage research (Good et al., 1992). We have used CLSM for quantitative evaluation of fluorescence signals in cartilage by localizing and quantifying insulin-like growth factor-1 (IGF-1) receptor expression in situ in cryostat sections of mouse articular cartilage (Verschure et al., 1994a). Analysis of distribution patterns in chondrocytes by conventional microscopical images did not allow precise localization of receptor immunoreactivity in relationship with the plasma membrane (Fig. 1). However, optical sectioning and CLSM analysis enabled us to distinguish IGF-1 receptor immunoreactivity on chondrocyte cell membranes from intracellular staining (Fig. 2). Two approaches were applied to quantify the low levels of fluorescence that were obtained after IGF-1 receptor staining. In the following sections, we describe our approach to analyze and quantify with CLSM in situ localization patterns of IGF-1 receptor in murine normal or arthritic intact patellae either or not after culture as well as in human normal and osteoarthritic articular cartilage samples. This approach yielded information from the chondrocyte in situ that could not be obtained either with conventional microscopy or biochemical methods.

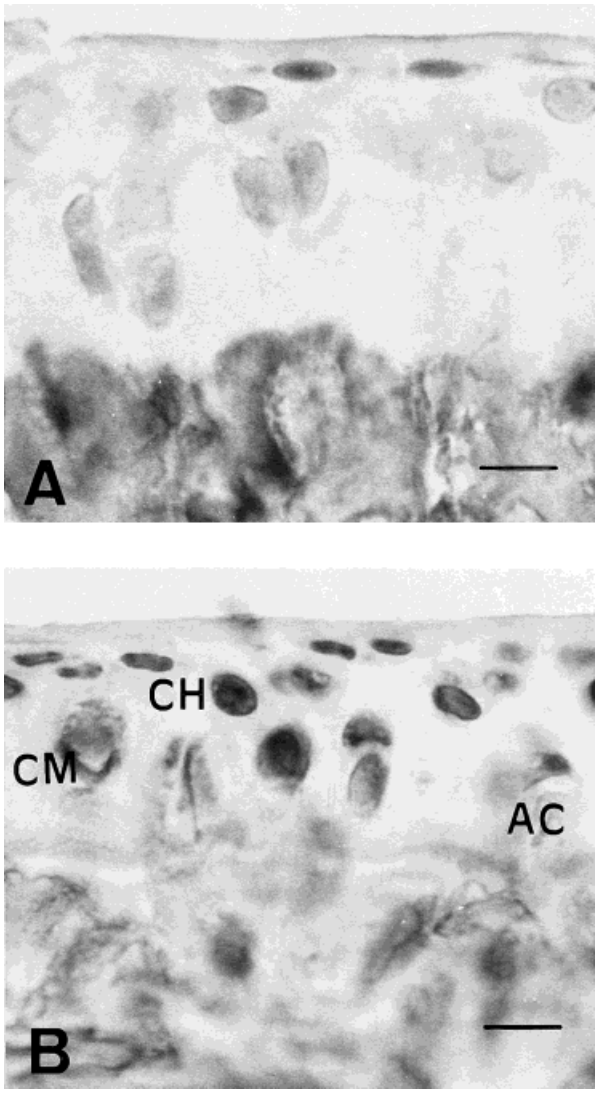


Fig. 1. IGF-1 receptor immunolocalization in normal mouse patellar cartilage. Cryostat sections were stained with the use of a rabbit polyclonal antibody against the α subunit of the IGF-1 receptor. Antigen-antibody binding is demonstrated by FITC labeling and evaluated with conventional fluorescence microscopy. **A:** Fluorescence microscopical image of chondrocytes (CH) within the articular cartilage (AC) were clearly stained, whereas the cartilage matrix (CM) was negative after staining with the antibody. Bar, 13 μm. **B:** Fluorescence microscopical image of control sections after staining with the use of non-immune rabbit serum shows low background staining. Bar, 13 μm (Reproduced with permission from Verschure, P.J., Van Marle, J., Joosten, L.A.B., and Van den Berg, W.B. (1994a) Localization and quantification of the insulin-like growth factor-1 receptor in mouse articular cartilage by confocal laser scanning microscopy. J. Histochem. Cytochem., 42:765–773).

IGF-1 RECEPTOR EXPRESSION IN NORMAL MOUSE ARTICULAR CARTILAGE

We have utilized CLSM to analyze the presence of IGF-1 type 1 receptors on chondrocytes in different zones of articular cartilage in mouse knee joints (Verschure et al., 1994a). IGF-1 is an essential anabolic growth factor for the regulation of chondrocyte biosynthetic functions, exerting its effects by binding to the IGF-1 type 1 receptor on chondrocyte plasma membranes (Froesch et al., 1985; Hascall et al., 1983; Isaksson, 1991; Le Roith and Riazada, 1989; Luyten et al., 1988; McQuillian et al., 1986; Neely et al., 1991; Osborn et al., 1989; Rechler and Nissley, 1990; Schalkwijk et al., 1989a; Trippel et al., 1988; Tyler, 1989). It is known that chondrocytes in different zones of cartilage vary with respect to their metabolic state (Byers et al., 1977; Aydelotte and Keutner, 1991; Maroudas et al., 1990; Rosier et al., 1988; Sampson and Cannon, 1986; Siczkowski and Watt, 1990). Receptor expression on membranes of isolated chondrocytes was analyzed with the use of affinity labeling (Dore et al., 1994). In this study, chondrocytes were isolated by enzymatic digestion of the cartilage and subsequently cultured with serum to be used for ^{125}I-IGF-1-binding studies. However, these biochemical techniques may introduce artifacts due to isolation procedures and give only information about the total receptor amount and do not provide topographic information. Therefore, we attempted to detect

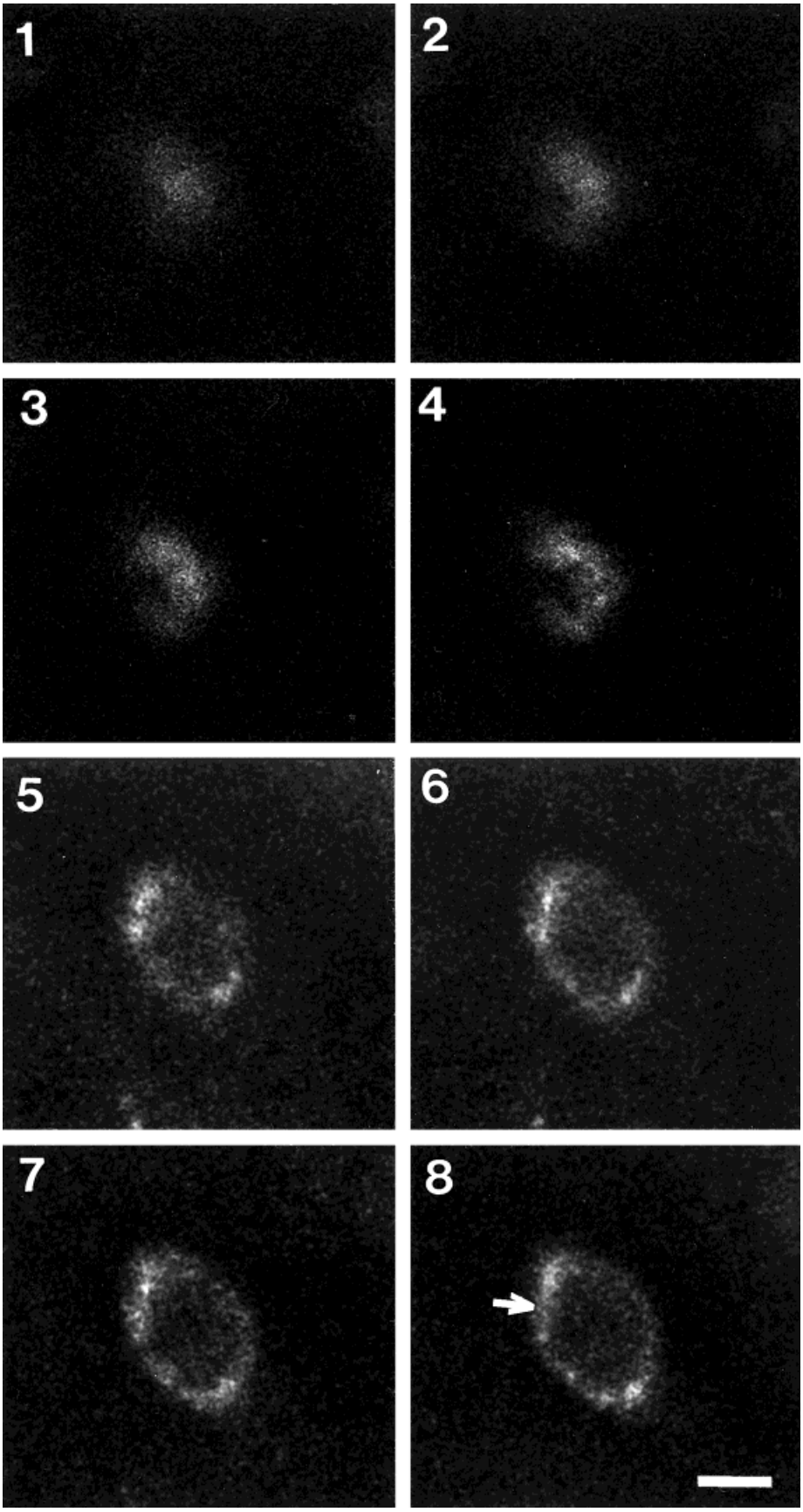


Fig. 2. CLSM evaluation of eight consecutive optical sections (1–8) of a chondrocyte recorded at 0.5 µm intervals after being immunohistochemically stained for the IGF-1 receptor. The chondrocyte membrane contained intense IGF-1 receptor immunoreactivity (arrow), whereas intracellularly a weak granular staining was displayed. Bar, 3.5 µm (Reproduced with permission from Verschure, P.J., Van Marle, J., Joosten, L.A.B., and Van den Berg, W.B. (1994a) Localization and quantification of the insulin-like growth factor-1 receptor in mouse articular cartilage by confocal laser scanning microscopy. J. Histochem. Cytochem., 42:765–773).

IGF-1 receptors with the use of immunofluorescence in cryostat sections of intact undecalcified normal mouse patellae (Rijntjes et al., 1979) using either a polyclonal antibody against the extracellular subunit of type 1 IGF-1 receptor (Oemar et al., 1991; Rosenzweig et al., 1990; Verschure et al., 1994a) or biotinylated IGF-1. Control sections were treated with non-immune rabbit serum or irrelevant biotinylated proteins with similar isoelectric point (PI) and molecular weight (MW) as biotinylated IGF-1. The immunolabelled sections were subjected to optical serial sectioning with a Leica CLSM providing images in the X-Y plane; images were recorded with intervals of 0.5 µm in the Z-direction. Chondrocytes located in the different zones of the cartilage were randomly selected with the use of a raster. For excitation of FITC, the 488-nm line of an argon-krypton laser was used and for emission a 510-nm dichroic mirror and a 530-nm band pass filter. The use of the antifading agent phenylenediamine largely prevented fading of the fluorescence caused by laser light excitation (Longin et al., 1993).

Conventional microscopical evaluation of fluorescent IGF-1 receptor staining revealed that sections showed a veil of aspecific fluorescence interfering with the specific staining of the receptor. Control sections showed low background staining and in sections stained for IGF-1 receptor, chondrocytes within the articular cartilage were positively stained for the receptor, whereas the cartilage matrix remained negative (Fig. 1). CLSM images demonstrated higher spatial resolution than traditional microscopical images. With the use of αIGF-1 receptor antibody intense immunoreactivity was found on the chondrocyte periphery and weak staining intracellularly (Fig. 2). CLSM analysis revealed that IGF-1 receptor staining at the chondrocyte cell surface was particularly present in chondrocytes of the middle and deeper zones of cartilage, whereas chondrocytes in the surface zone displayed a distinctly less bright peripheral signal (Fig. 3). On the other hand, high IGF-1 receptor immunoreactivity was found intracellularly in chondrocytes located in the surface zone of cartilage.

Evaluation of optical sections revealed that staining with biotinylated IGF-1 gave consistently higher amounts of intracellular fluorescence intensity after staining with biotinylated IGF-1 than with antibodies against the IGF-1 receptor, although the exact intracellular localization, e.g. localization in endoplasmatic reticulum or other cellular compartments, could not be established (Fig. 4). An explanation for this phenomenon might be that biotinylated IGF-1 may also bind to IGF-1-binding proteins (IGFBP). IGFBPs show binding affinity for IGF-1 which is comparable to that of IGF-1 for the IGF-1 type 1 receptor (Baxter et al., 1993). Therefore, our observations seem to indicate that when labeled growth factors are used, caution should be taken when drawing conclusions about the expression of a particular type of receptor, because this method may not solely detect the receptor only. Along this line it is noteworthy that labeled growth factors are frequently used to detect the growth factor receptor in cancer research, and that enhanced expression is often interpreted as an indication of growth. Future studies with anti-receptor antibodies would establish whether this is the case.

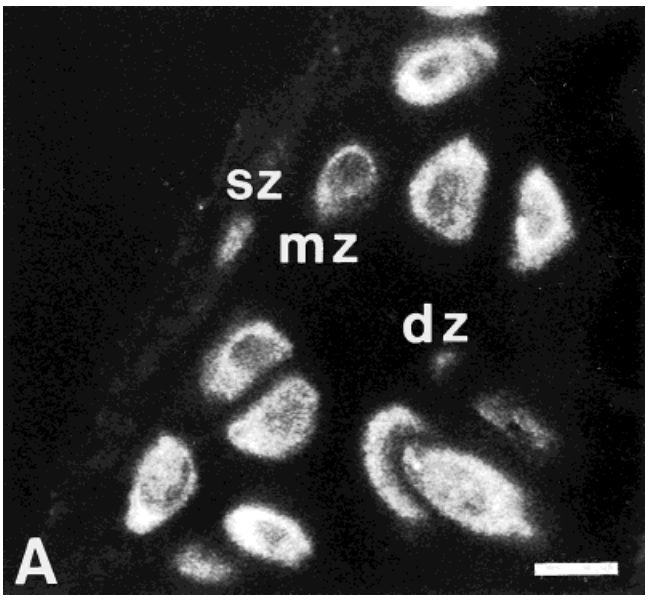


Fig. 3. Immunolocalization of IGF-1 receptors on chondrocytes localized in different zones of mouse cartilage and visualized by CLSM. **A:** Overview of surface, middle and deeper zones of articular cartilage. Chondrocytes fixed in the middle zone (mz) and deeper zones (dz) show bright positive staining at the chondrocyte periphery. Chondrocytes in the surface zone (sz) show fluorescence that is similar to background fluorescence. Bar, 8 µm. **B:** Higher magnifications of IGF-1 receptor immunolocalization on chondrocytes located in the different zones of mouse cartilage as visualized by CLSM. The cell surface of chondrocytes located in the middle zone (MZ) and deeper zone (DZ) show bright positive staining, while staining of chondrocytes in the surface zone (SZ) display intracellular staining only. Bars, 1.5 µm. (Reproduced with permission from Verschure, P.J., Van Marle, J., Joosten, L.A.B., and Van den Berg, W.B. (1994a) Localization and quantification of the insulin-like growth factor-1 receptor in mouse articular cartilage by confocal laser scanning microscopy. J. Histochem. Cytochem., 42:765–773).

Quantification of immunofluorescence indicating IGF-1 receptor expression was performed in two ways (Verschure et al., 1994a). One approach was point measurement by determining in the sections of a series of optical sections the fluorescence intensity of eight pixels evenly distributed over the chondrocyte periphery. The mean fluorescence intensity of these pixels was taken as a measure of immunoreactivity. To assess the contribution of autofluorescence and aspecific binding, the fluorescence signal in sections stained with control non-immune serum was measured as well. The second approach was area measurement by determination of fluorescence intensity of the chondrocyte periphery by manually setting a window over the chondrocyte periphery with a width of 0.2 µm. The CLSM instrument software provides the averaged intensity ($\bar{x}/\mu m^2$) as found in the window. Again, nonspecific fluorescence was determined in control sections. In general, both methods yielded comparable and reproducible results, demonstrating the validity of the quantitative approach. The amount of background fluorescence in the different cartilage layers as determined with both methods was relatively high in comparison with total fluorescence.

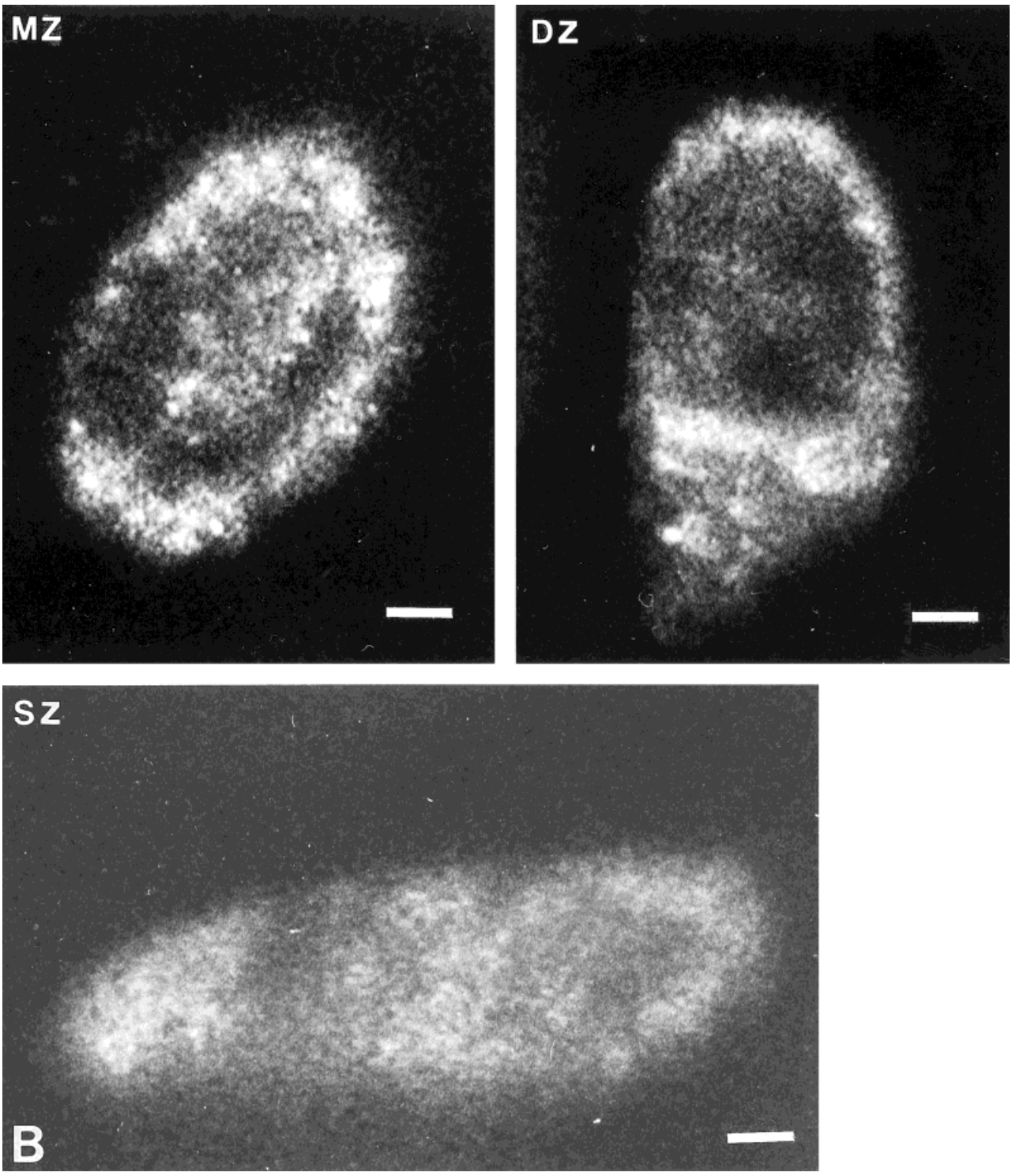


Fig. 3. (Continued.)

This is an inherent problem of low levels of antigen and high levels of autofluorescence in cartilage specimens. However, both quantification methods clearly demonstrated that the fluorescence intensity of chondrocytes located in middle and deeper zones of the cartilage was significantly higher than that of chondrocytes located in the surface zone (Fig. 5). The fluorescence intensity signals of chondrocytes in the surface zones did not

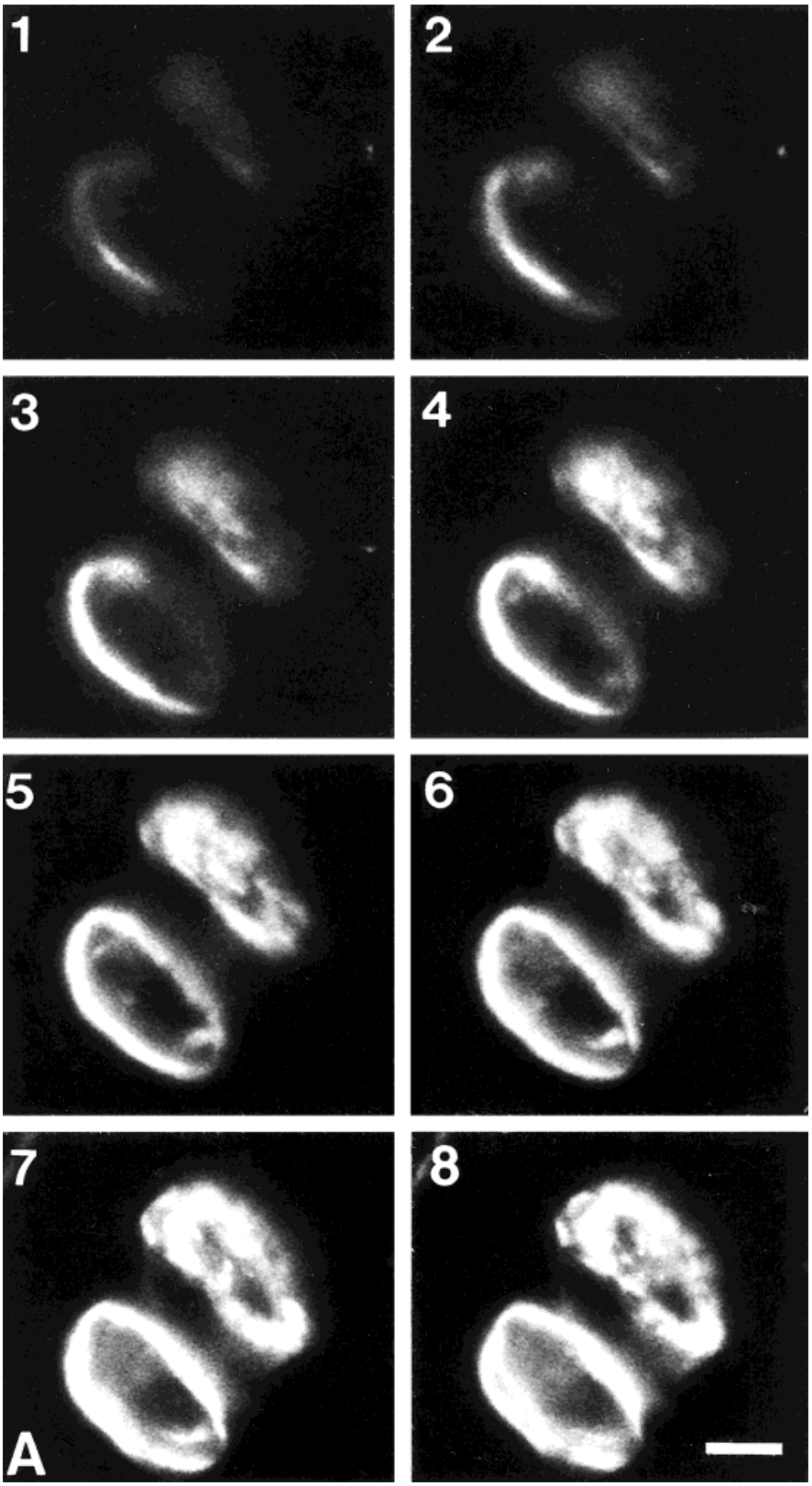


Fig. 4. CLSM evaluation of the localization of IGF-1 receptor in mouse normal articular cartilage stained with either a polyclonal antibody against IGF-1 receptor (A) or with biotinylated IGF-1 (B) in consecutive optical sections (1–8) through the depth of the chondrocyte. Bars, 4 µm. **A:** Chondrocytes stained with a polyclonal antibody against the α subunit of the receptor showed that the cell membrane contained intense IGF-1 receptor immunoreactivity, whereas intracellularly a weak granular staining was displayed. **B:** Chondrocytes stained with biotinylated IGF-1 showed a higher amount of intracellular fluorescence in comparison with an antibody against the IGF-1 receptor.

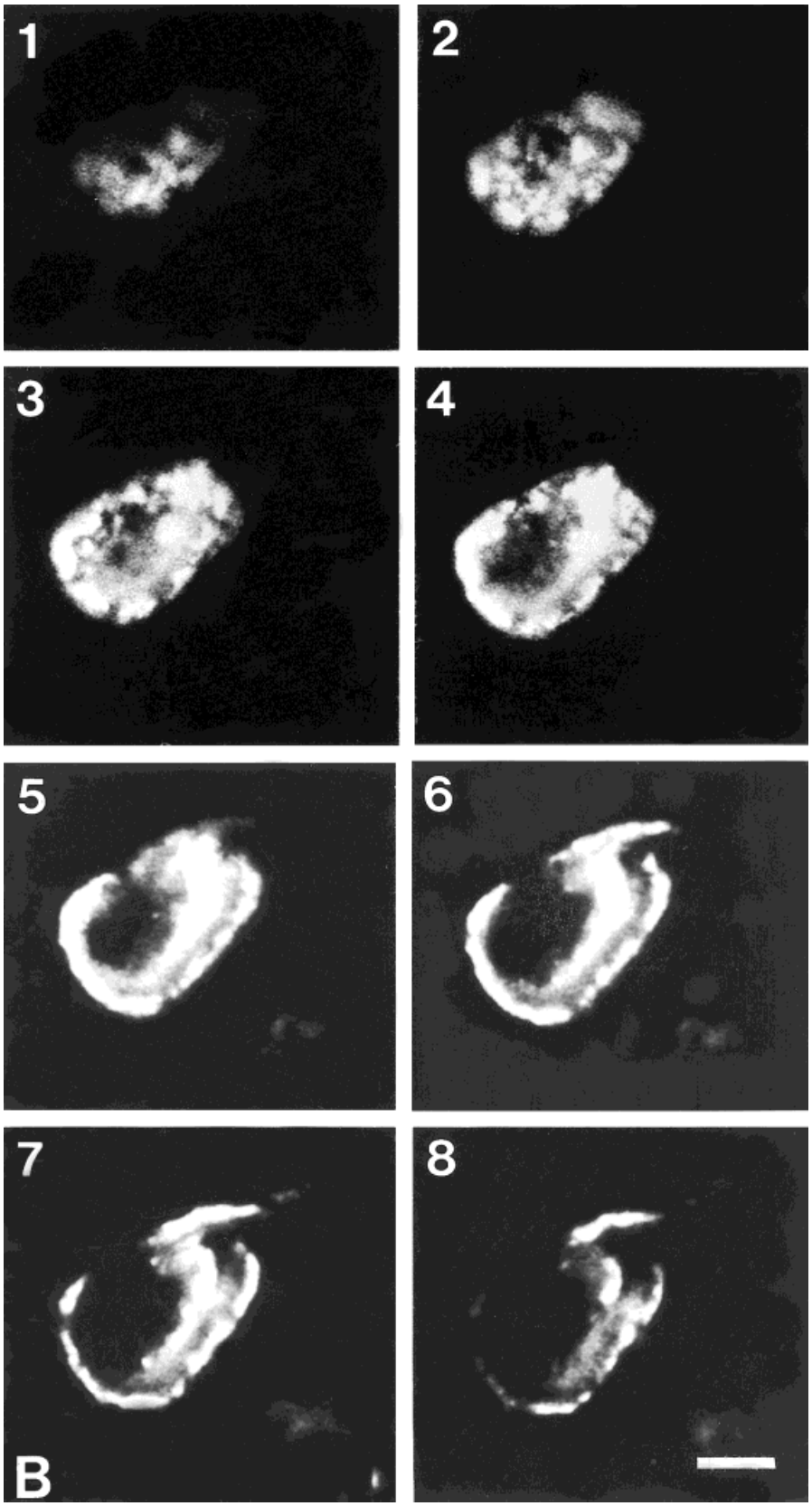


Fig. 4. (Continued.)

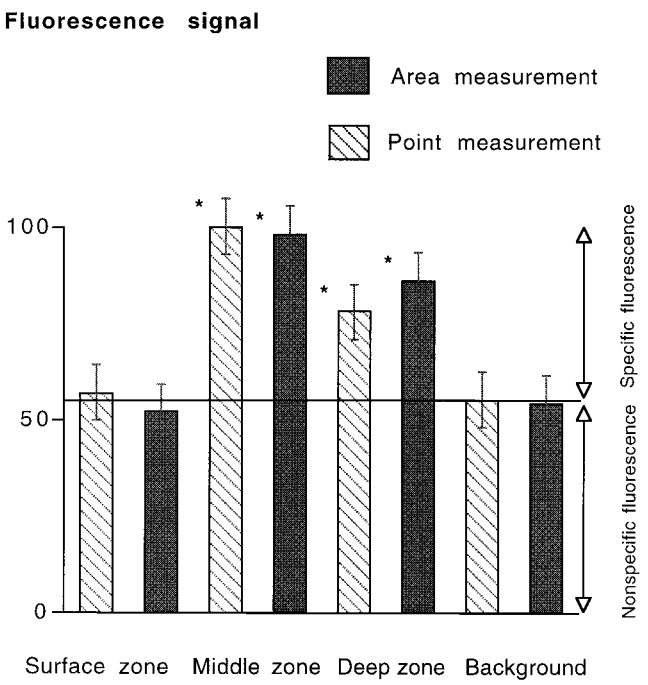


Fig. 5. Quantification of the fluorescence signal of IGF-1 receptor immunoreactivity on chondrocyte membranes as detected in confocal images after incubation with polyclonal anti-IGF-1 receptor antibody. Nonspecific fluorescence is assessed in images of chondrocytes incubated with non-immune rabbit serum. Point measurement of fluorescence intensity on chondrocytes located in the different zones of the cartilage, i.e. the surface, middle and deeper zones, is compared with area measurement. Fluoresence intensity was measured in 10 chondrocytes in each cartilage layer in two serial sections. The data are the mean ± SEM of four independent experiments, expressed as percentage of the highest value of each experiment. *Mean value is significantly different from the mean value in the surface zone ($P < 0.05$) by Wilcoxon rank test. (Reproduced with permission from Verschure, P.J., Van Marle, J., Joosten, L.A.B., and Van den Berg, W.B. (1994a) Localization and quantification of the insulin-like growth factor-1 receptor in mouse articular cartilage by confocal laser scanning microscopy. J. Histochem. Cytochem., 42:765–773).

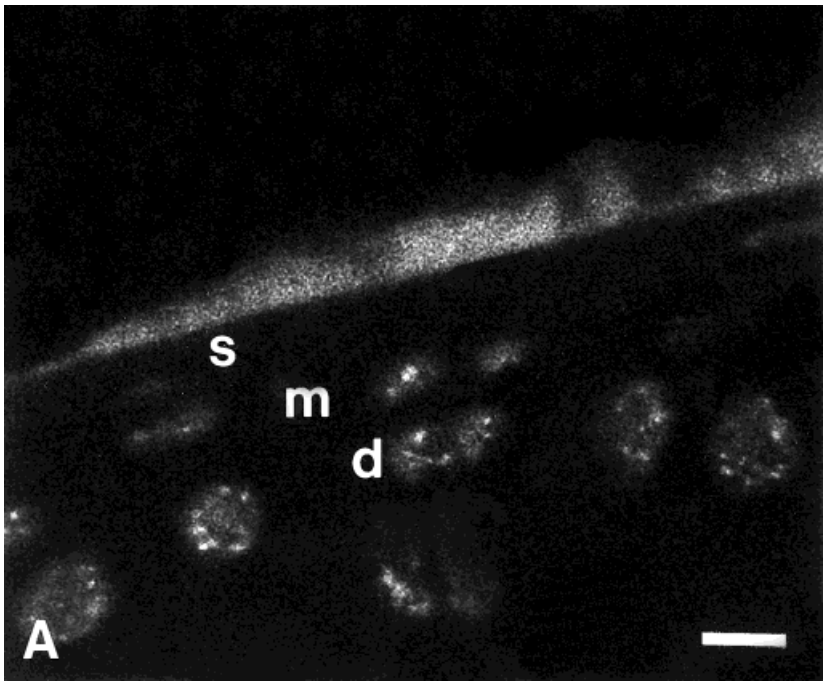
raise above levels of control fluorescence (Fig. 5). The application of CLSM was essential to find these differences in receptor expression in chondrocytes in the different zones of intact murine articular cartilage, indicating that probably IGF-1 stimulation and metabolic activity of chondrocytes in these layers is heterogeneous.

IGF-1 RECEPTOR EXPRESSION IN DISEASED MOUSE ARTHRITIC CARTILAGE

In knee joints with experimentally induced arthritis the articular cartilage is severely destroyed, which is thought to be evoked by accelerated degradation of matrix components accompanied by inhibition of chondrocyte proteoglycan synthesis and synthesis of proteoglycan molecules with inferior integrity (Sandy et al., 1980; Van den Berg et al., 1981; Verschure et al., 1994b). Moreover, there is compelling evidence that lack of anabolic signaling might contribute to the decrease in chondrocyte proteoglycan synthesis. In contrast with the ability of IGF-1 to sustain chondrocyte proteoglycan synthesis in normal murine cartilage during culture, chondrocytes from experimentally induced arthritic knee joints do not respond to IGF-1 in culture (Schalkwijk et al., 1989b; Van den Berg et al., 1989; Verschure et al., 1994c). We hypothesized that nonresponsiveness to IGF-1 stimulation as observed in experimentally induced arthritis may reflect reduced levels of chondrocyte IGF-1 receptors during the inflammatory response in the joint. With the use of CLSM we were able to demonstrate that after induction of arthritis, IGF-1 receptor expression declines during a short period of time in all cartilage layers (6–24 hours after induction of arthritis) (Fig. 6). In later stages of arthritis, amounts and localization patterns of IGF-1 receptor in the various cartilage layers were not different from those in control cartilage (Fig. 6). Thus at these later stages of arthritis, a significant decrease in IGF-1 receptor expression could not be found when chondrocytes have lost their capacity to respond to IGF-1 and chondrocyte synthesis is low. This implies that in these stages of arthritis either a defect in the expressed receptor or a disturbance in post-receptor signaling is responsible for the lack of IGF-1 stimulation. Furthermore, it shows that IGF-1 receptor expression does not necessarily correlate with chondrocyte IGF-1 stimulation. Apparently, data from studies of receptor expression without evaluation of the biological responses as triggered by receptor-ligand binding should be interpreted with caution.

IGF-1 RECEPTOR EXPRESSION IN CULTURED MOUSE CARTILAGE

When normal mouse patellar cartilage is cultured in the absence of serum or growth factors proteoglycan synthesis declines rapidly. Proteoglycan synthesis is stimulated when cartilage is cultured in the presence of IGF-1 (Schalkwijk et al., 1989b; Verschure et al., 1994c). A shift in metabolic activity in the different cartilage layers has been found after prolonged culture of human cartilage explants (Lafeber et al., 1992, 1993; Meachim et al., 1965). We have performed CLSM analysis to measure shifts in IGF-1 receptor expression, in normal patellae and patellae isolated from knee joints in very early stages of experimentally induced arthritis in mice after 24 hours of culture. Culture in the absence of IGF-1 caused a striking loss of receptors in all layers of normal and arthritic cartilage. Only a low level of expression remained at the tendon insertion sites. Normal cartilage showed enhanced expression of IGF-1 receptors in both middle and deeper zones of the cartilage after culture in the presence of IGF-1. However, chondrocytes in the surface zones, which normally show low expression, now displayed intense expression of IGF-1 receptors after culture in the presence of IGF-1 (Fig. 7). The effect of 24-hour culture in the presence of IGF-1 was strikingly different in arthritic cartilage isolated 12 hours after induction of arthritis. The pattern was highly heterogeneous with extremely high receptor expression in some chondrocytes in the middle and deeper zones of the cartilage, while chondrocytes in the surface zones still showed a total lack of receptor expression (Fig. 7). These data indicate that shifts as observed in proteoglycan synthesis after culture may originate from an increase in receptor expression of chondrocytes located in the various cartilage zones. It also underlines that receptor expression may change considerably after culture and that data on receptor



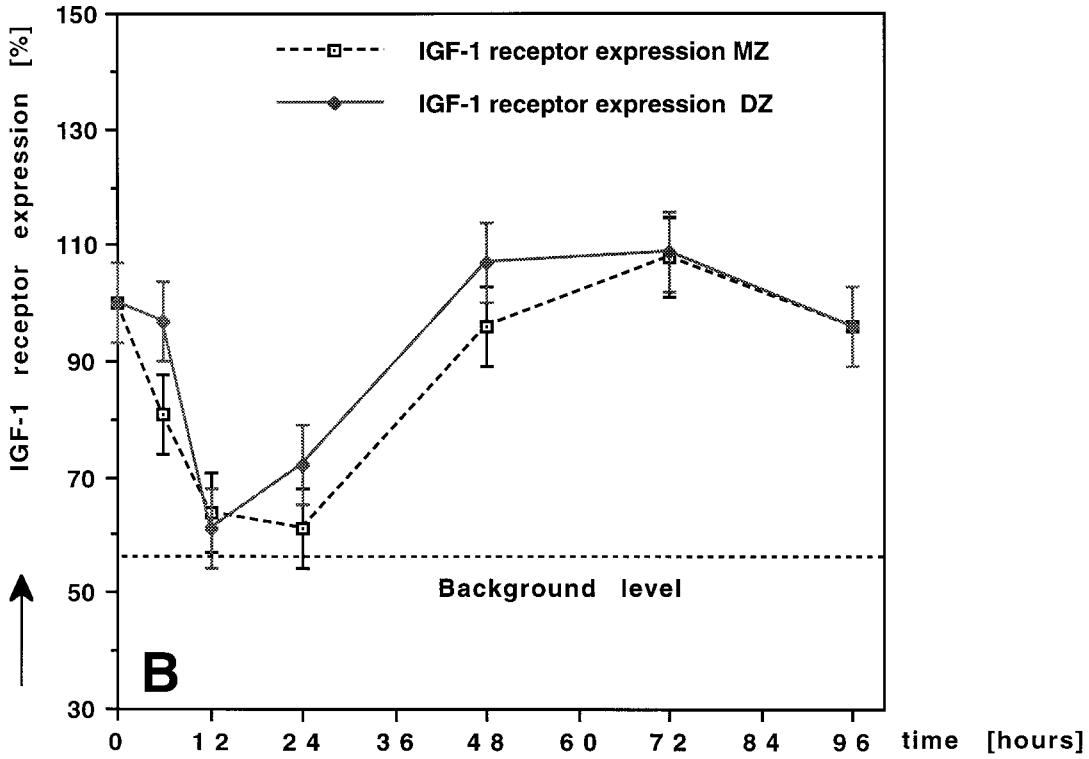


Fig. 6. IGF-1 receptor immunolocalization in cryostat sections of mouse patellar cartilage at various phases of zymosan-induced arthritis and visualized by CLSM. **A:** At 12 hours after zymosan-induced arthritis very weak IGF-1 receptor immunoreactivity was observed in the surface (S), middle (M) and deeper (D) zones. Bar, 8 µm. **B:** Quantification with the use of digitized CLSM images of IGF-1 receptor immunoreactivity in mouse patellar cartilage at various stages of zymosan-induced arthritis. Fluorescence intensity was determined at the cell membranes of chondrocytes located in the middle zone (MZ) and deeper zones (DZ) of cartilage. The data are the mean ± SEM of three independent experiments and expressed as percentage of the intensity as detected in chondrocytes located in the same cartilage zones of normal cartilage. *$P < 0.05$, by Wilcoxon rank test.

expression as obtained from either cultured chondrocytes or cultured cartilage specimens should be interpreted with great care; their relevance to the situation in vivo may be limited.

IGF-1 RECEPTOR EXPRESSION IN HUMAN ARTICULAR CARTILAGE

Chondrocyte metabolism is elevated in human osteoarthritic (OA) cartilage, which can be explained as an attempt to repair cartilage damage and to limit net cartilage loss (Bullough, 1988; Dieppe, 1990; Hardingham and Bayliss, 1990; Huskinsson, 1988). In line with this, levels of IGF-1, IGF-1 mRNA and IGF-1 receptor expression are also increased in human OA cartilage (Dore et al., 1994; Middleton and Tyler, 1992). We examined IGF-1 receptor expression on the chondrocyte membrane in different cartilage zones of normal and osteoarthritic human cartilage with CLSM and compared it with chondrocyte proteoglycan synthesis as determined by ^{35}S-sulfate incorporation and proteoglycan content by Safranin O staining (Rosenberg, 1971). Cartilage explants, obtained either by obduction from human knee joints within 18 hours after death without a clinical history or from patients undergoing knee arthroplasty, were defined according to established criteria as "normal" or "mild" to "severe" OA cartilage on the basis of histology (Lafeber et al., 1992; Mankin and Brandt, 1984). Both IGF-1 receptor staining (Fig. 8A), Safranin O staining (Fig. 9A) and ^{35}S-sulfate incorporation (Fig. 9C) were low in the surface zone of normal cartilage explants. Strong IGF-1 receptor staining (Fig. 8B) and high ^{35}S-sulfate incorporation (Fig. 9C) and Safranin O staining (Fig. 9A) were found in the middle and deeper zones of human normal cartilage. A similar distribution pattern of receptor staining, proteoglycan synthesis and proteoglycan content were found in cartilage explants defined as "mild OA." "Severe OA" cartilage samples showed a distinct heterogeneity with respect to both intensity and distribution patterns of IGF-1 receptors between the different donors. In some OA donors all cartilage layers showed very low or even lack of staining (Fig. 8C,D), whereas in other donors very high receptor staining was observed especially in the deeper zones of the cartilage (Fig. 8E,F). All cartilage explants of these OA donors showed severe pathological deviations histologically (Fig. 9B,D). Superficial zones of the cartilage were strikingly damaged and fibrillated. A strong reduction of Safranin O staining (Fig. 9B) and high ^{35}S-sulfate incorporation (Fig. 9D) was found. Cell clusters were present in the surface zones as well as close to the lesions perpendicular to the surface. These results demonstrate clearly that the CLSM allows comparison of receptor localization with site-specific synthesis and content.

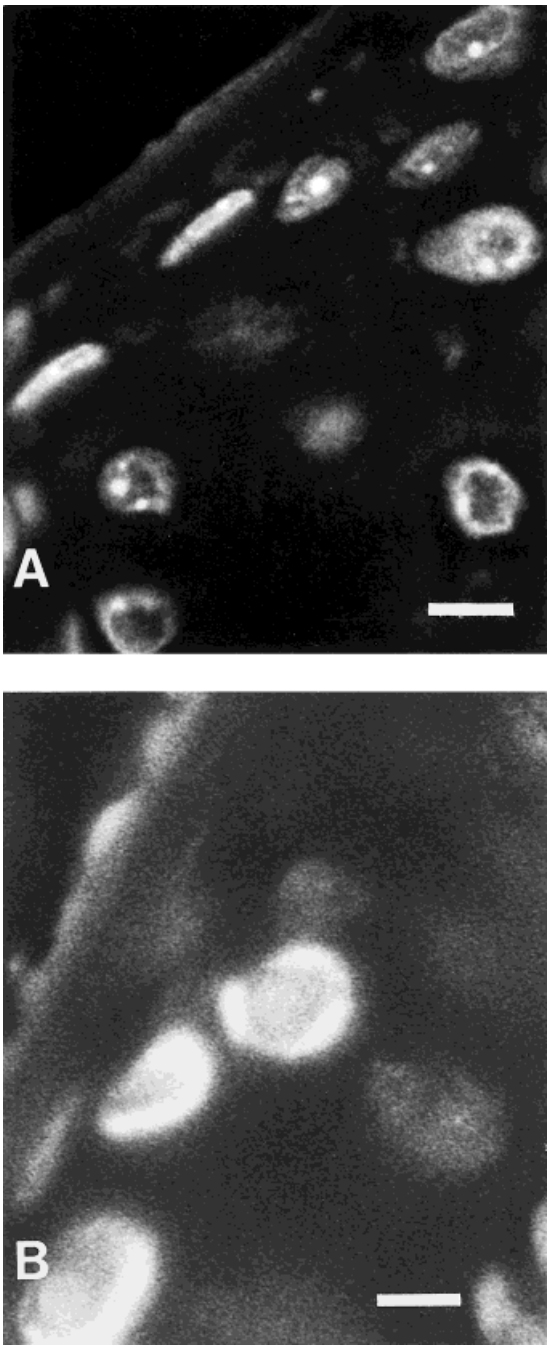


Fig. 7. IGF-1 receptor immunolocalization in cryostat sections of mouse patellar cartilage cultured for 24 hours in the presence or absence of IGF-1. Normal cartilage and cartilage isolated 12 hours after induction of zymosan-induced arthritis were analyzed by CLSM. **A:** Normal cartilage cultured in the presence of IGF-1 showed the presence of receptors on chondrocytes in the surface zone of cartilage and increased receptor staining in the middle and deeper zones. Bar, 8 µm. **B:** Cartilage from knee joints during an early phase of arthritis cultured in the presence of IGF-1 showed enhanced receptor staining in some chondrocytes located in the middle and deeper zones of the cartilage. Bar, 4 µm.

FINAL CONSIDERATIONS AND FUTURE APPLICATIONS OF CLSM IN CARTILAGE RESEARCH

We have considered the potential of CLSM in the field of cartilage research. The exact mechanisms underlying cartilage destruction and eventually joint failure during rheumatoid arthritis (RA) and osteoarthritis (OA) still need to be unraveled. There is substantial evidence to suggest that interleukin-1 acts as a key mediator in the inhibition of chondrocyte synthetic activity (Benton and Tyler, 1988; Hardingham et al., 1992a; Van de Loo and Van den Berg, 1990; Van de Loo

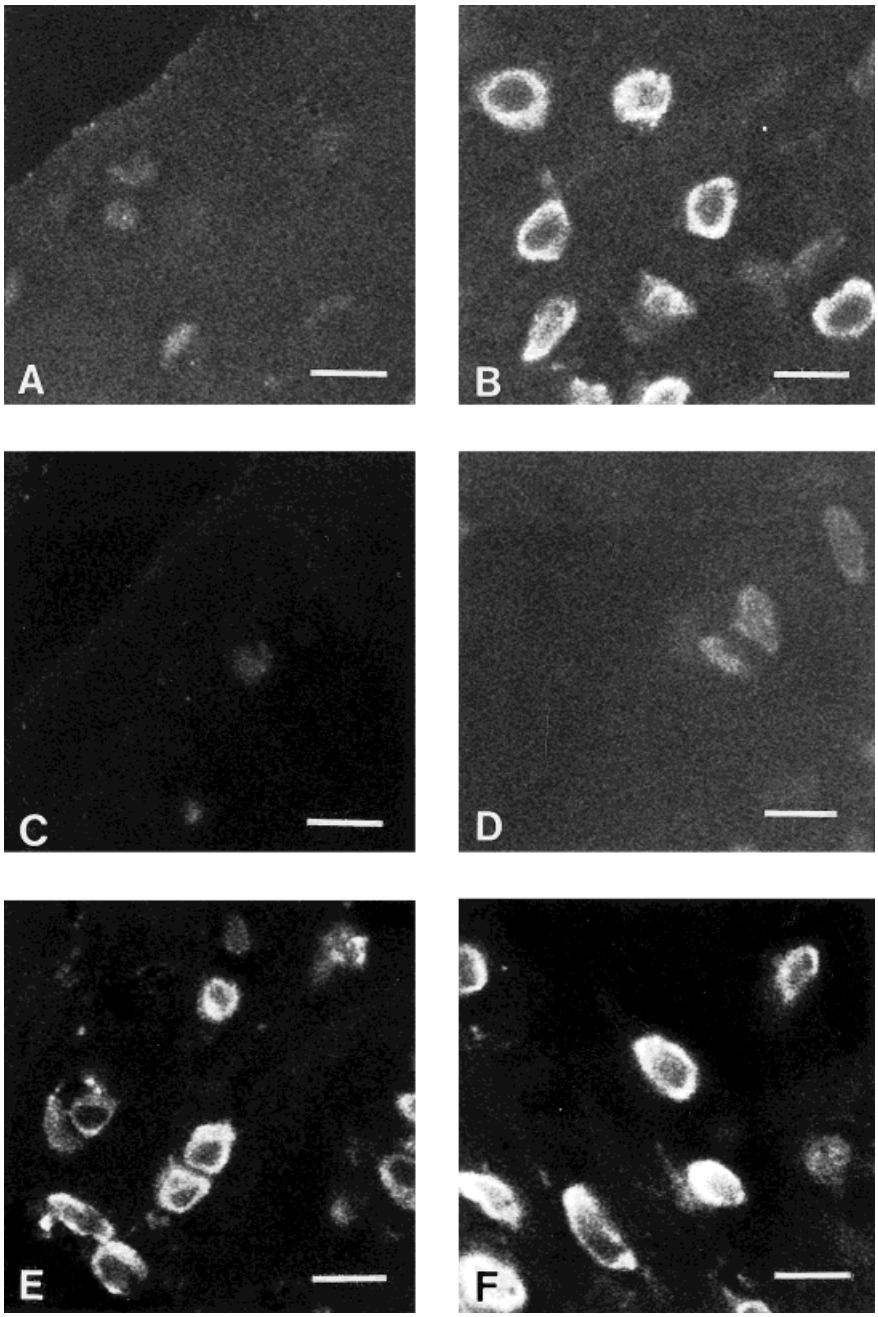


Fig. 8. IGF-1 receptor expression was determined by immunolocalization and CLSM analysis in sections of human normal (A) and "severe" osteoarthritic cartilage (B,C). In the surface zone low amounts of receptor were observed (**A**) whereas IGF-1 receptors were mainly found in the middle and deeper zones of the cartilage (**B**). The amount of IGF-1 receptors showed marked donor-dependent heterogeneity in sections of "severe" osteoarthritic human cartilage. In some OA donors all cartilage layers showed very low or undetectable levels of staining (**C,D**). In other donors, high receptor staining was observed especially in the deeper zones of the cartilage (**E,F**). Bars, 8.5 μm.

et al., 1992; Verschure and Van Noorden, 1990), although the exact roles of growth factors in these processes are still far from understood.

Quantification of chondrocytes labeled in situ with immunofluorescence implies measuring their response in an environment characterized by high autofluorescence and high background staining. Although autofluorescence can be reduced by adequate optical filtering and the choice of an appropriate probe, it remains a complicating factor in cartilage research. Background fluorescence is a problem due to the properties of the matrix and the presence of various binding proteins. In case of probes that bind to or in the vicinity of cellular membranes an additional problem arises concerning cellular membrane-related fluorescence at the periphery of chondrocytes. Using CLSM, thin optical sections

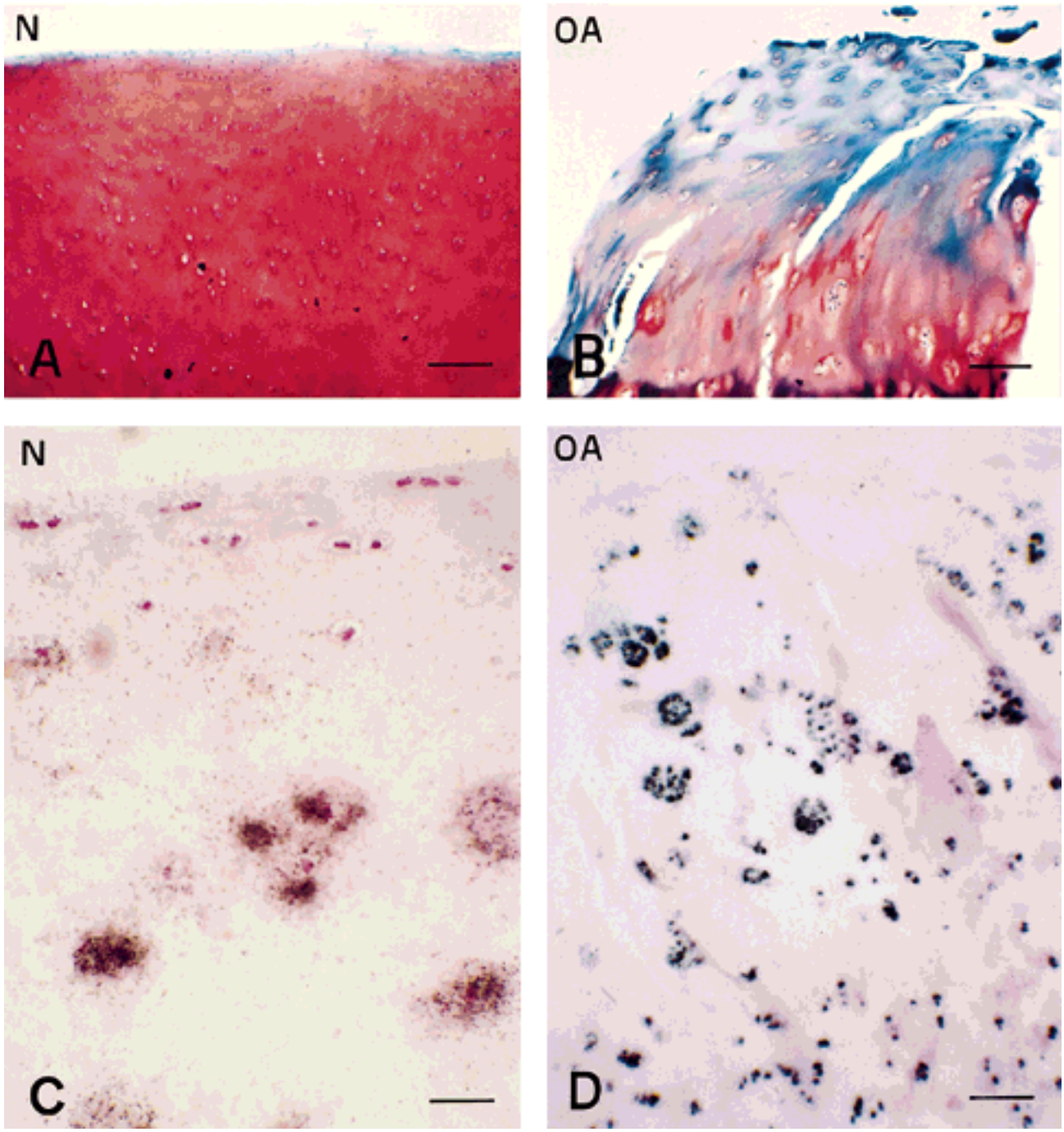


Fig. 9. Proteoglycan content was detected by Safranin O staining (A,B) and proteoglycan synthesis levels were analyzed by in situ autoradiography after ^{35}S-sulfate incorporation (C,D) in sections of human normal cartilage (N) and cartilage that was defined as "severe" osteoarthritic (OA). **A:** Low levels of Safranin O staining were noted in the surface zone of human normal cartilage. Bar, 60 µm. **B:** Superficial zones of cartilage samples of "severe" osteoarthritic cartilage were considerably damaged and fibrillated. Cell clusters were present in the surface zone and perpendicular to the surface. Safranin O staining was strongly reduced. Bar, 30 µm. **C:** ^{35}S-sulfate incorporation was mainly found in the middle and deeper zones of normal human cartilage, whereas the ^{35}S-sulfate incorporation in the surface zone was very low. Bar, 30 µm. **D:** In situ autoradiography after ^{35}S-sulfate incorporation showed a relatively high level of proteoglycan synthesis in "severe" osteoarthritic human cartilage. Bar, 60 µm.

can be made. Consequently, interference of over- and underlying matrix can be reduced significantly. With the use of CLSM the measuring volume of a compartment of interest can be optimized by selecting for each chondrocyte the ideal cross section, which ensures that the compartment of interest is displayed at best. These

properties of confocal imaging, i.e. optical isolation of chondrocytes from their environment and optimizing the amount of cellular constituents to be measured, allows reliable measurement of immunofluorescence in such a way that was not possible in any other way. Moreover, CLSM analysis of metabolic events in living cartilage explants will become increasingly important in cartilage research. In conclusion, CLSM imaging will attribute to a better understanding of 2D and 3D morphology of cartilage in relation to physiological responses.

The application of CLSM gave us the tools to correlate chondrocyte growth factor receptor expression with chondrocyte biological response. As shown for the IGF-1 receptor, the presence of receptor may at best be an indication for receptor activity. The actual receptor, signal transduction, e.g. phosphorylation patterns, should be investigated in concert. Regarding future perspectives, application of CLSM may become of vital importance to study in situ immunolocalization in intact cartilage of factors involved in joint pathology. The availability of antibodies suited for immunohistochemistry will determine the rate of progress in these studies. Our CLSM studies indicated that caution should be taken when drawing conclusions from receptor expression studies without evaluating biological responses triggered by receptor binding.

ACKNOWLEDGMENTS

The authors thank Henk van Veen, Jan Peeterse and Rob Lutgerhorst for excellent technical assistance. Figures 1, 2, 3 and 5 are reproduced with permission from Verschure PJ, Van Marle J, Joosten LAB, Van den Berg WB (1994). Localization and quantification of the insulin-like growth factor-1 receptor in mouse articular cartilage by confocal laser scanning microscopy. *J. Histochem. Cytochem.* 42:765–773. This study was supported by TNO.

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