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LOCALIZATION OF INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR IN HUMAN NORMAL AND OSTEOARTHRITIC CARTILAGE IN RELATION TO PROTEOGLYCAN SYNTHESIS AND CONTENT

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SUMMARY

Insulin-like growth factor-1 (IGF-1) plays a key role in the regulation of chondrocyte proteoglycan (PG) metabolism. We investigated whether chondrocyte PG synthetic activity correlates with the presence of chondrocyte IGF-1 receptor in the surface, middle and deeper zones of normal human articular cartilage and in cartilage known to display a shift in chondrocyte metabolism, i.e. cultured cartilage or osteoarthritic (OA) cartilage. Cartilage specimens were obtained post mortem from human knees within 18 h after death from donors without known clinical OA history. The samples were taken from macroscopically normal looking regions as well as from damaged regions with osteoarthritic appearance, yielding a range of OA grades from mild to moderate and severe OA. We examined chondrocyte PG synthesis by *in situ* autoradiography of incorporated [³⁵S]sulphate and chondrocyte IGF-1 receptor localization by immunohistochemistry, followed by confocal laser scanning microscopical (CLSM) analysis in the same cartilage samples. In normal cartilage, both the amount of chondrocyte PG synthesis and the level of chondrocyte IGF-1 receptor localization are at low levels in the surface zone chondrocytes, but both are high in middle and deeper zone chondrocytes. Furthermore, after culture, the increase in chondrocyte PG synthesis in the surface layer coincides with increase in IGF-1 receptor expression. However, in mild OA particularly high levels of chondrocyte synthetic activity were found in the upper cartilage layer, whereas IGF-1 receptor expression was low in this layer, suggesting that factors other than IGF-1 are involved. High chondrocyte PG synthetic activity and chondrocyte IGF-1 receptor staining were found in the upper and deeper layers of moderate OA cartilage, whereas both low levels of chondrocyte activity as well as IGF-1 receptors were observed in cases of severe OA. Our data indicate that IGF-1 displays cellular heterogeneity in chondrocyte stimulation in the various cartilage zones in normal cartilage. Clear zonal correlation is lost in OA cartilage, and patterns of chondrocyte IGF-1 receptor expression and PG synthesis vary with the stage of OA.

KEY WORDS: Chondrocyte, Cartilage, OA, IGF-1 receptor, PG synthesis, *In situ*, CLSM, Zonal analysis.

IN degenerative joint diseases such as osteoarthritis (OA), the equilibrium between the degradation and synthesis of matrix molecules is disturbed [1-3]. Increased proteoglycan (PG) metabolism of chondrocytes in OA cartilage, which is probably an attempt to repair damage, is unable to overcome the increased losses of PG and leads to focal depletion of PG, and eventually loss of cartilage and changes in underlying bone [4]. The biosynthetic function of chondrocytes is regulated by the complex effects of circulating and locally produced growth factors. Understanding the influence of growth factors on chondrocyte metabolism during normal physiological regulation of cartilage remodelling, or during pathological cartilage degradation, will be a basis for successful intervention in cartilage degeneration.

Insulin-like growth factor-1 (IGF-1) is an important anabolic stimulus that regulates chondrocyte PG biosynthesis during growth and development, cartilage

hypertrophy and repair during adult life [5-10]. IGF-1 stimulation of intact articular cartilage results in a significant increase in [³⁵S]sulphate uptake, i.e. chondrocyte PG synthesis [11-14]. IGF-1 exerts its effect by high-affinity binding with specific IGF-1 type 1 receptors [15-18].

Chondrocytes in articular cartilage are not a uniform population of cells. They vary in metabolic activity and function in the different cartilage zones [19-23]. Moreover, there are indications that chondrocyte PG metabolism alters in the different cartilage zones in cartilage diseases [24-27]. In human OA, high levels of IGF-1 and IGF-1 mRNA have been found in cartilage, especially in chondrocytes of the surface zone [28]. Furthermore, Dore *et al.* [29] showed abundant immunohistochemical staining of the IGF-1 receptor in the upper two-thirds of human OA cartilage.

In the present study, we examined articular cartilage obtained from human knees within 18 h after death, from donors without a clinical history of OA. The samples were classified, yielding normal cartilage and a range of OA grades from mild via moderate to severe OA. The aim of our study was to investigate whether a correlation exists between chondrocyte synthetic activity, chondrocyte IGF-1 receptor expression and

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cartilage PG content in the surface, middle and deeper zones of normal cartilage. Moreover, we analysed whether the well-known shift in chondrocyte PG synthesis in cultured cartilage also coincides with a shift in chondrocyte IGF-1 receptor expression. Finally, we investigated whether in the various grades of OA cartilage a zonal correlation also exists between the chondrocyte PG synthetic rate and IGF-1 receptor expression.

MATERIALS AND METHODS

Cartilage

Cartilage sampling was performed as previously described [24, 30]. Briefly, cartilage samples were obtained, post mortem, from the central part of human femoral knee condyles within 18 h after death from donors without a known clinical OA history. Within 1 h post-dissection, the cartilage samples were cut, excluding the underlying bone. The donors are described in Table I; there were two females and eight males, ranging in age from 59 to 79 yr with a mean age of 73 ± 6 (S.E.M.) yr. From each donor, samples from macroscopically normal regions or pathological looking 'osteoarthritic' regions were taken. The pathological regions occur very locally, only affecting some areas of the cartilage. Cartilage samples from 'osteoarthritic' regions were classified after tissue processing and histological staining according to the slightly modified criteria of Mankin [4, 24] (Table I). Samples from pathological regions were compared with cartilage obtained from patients undergoing knee arthroplasty; cartilage samples characterized as 8 or > 8 on the Mankin scale showed very similar staining patterns as were observed in cartilage from patients undergoing knee arthroplasty. The cartilage from normal looking regions showed only minor variation in responses, i.e. [^{35}S]sulphate incorporation, Safranin O staining and IGF-1 receptor immunostaining (data not shown). Comparison of samples from normal looking regions with joints showing no pathology has been described previously [30].

Experimental design

We aimed to analyse the cartilage PG content, the chondrocyte PG synthesis and chondrocyte IGF-1 receptor localization of the same cartilage area. Therefore, the isolated cartilage specimens were divided into three smaller cartilage pieces. Subsequently, one piece was used for histological Safranin O staining, one for autoradiographic localization of incorporated [^{35}S]sulphate and one for immunohistochemical localization of IGF-1 receptor followed by confocal laser scanning microscopical (CLSM) analysis. Moreover, cartilage specimens were first labelled with [^{35}S]sulphate, frozen in liquid N_2 , and autoradiographic localization of [^{35}S]sulphate and IGF-1 receptor immunostaining were analysed in serial sections.

Light microscope analysis

Cartilage samples were fixed in phosphate-buffered 4% formalin (pH 7.0) containing 2% sucrose. Standard processing of the tissue in an automatic tissue-processing apparatus was followed by embedding the samples in paraffin wax. Histological sections were stained with Safranin O and Fast Green or haematoxylin.

Culture technique

The cartilage slices were cut into square pieces, weighed (with an accuracy of ± 0.1 mg) and cultured individually at random for 4 days in round-bottomed, 96-well microtitre plates (200 μl culture medium/well, 37°C, 5% CO_2 in air). The culture medium was Dulbecco's modified Eagle's medium (DMEM; Gibco, s'Hertogenbosch, The Netherlands, 074-01600; 0.81 mM SO_4^{2-} ; 24 mM NaHCO_3) supplemented with ascorbic acid (0.85 mM), glutamine (2 mM), penicillin (100 U/ml) and streptomycin sulphate (100 U/ml) and 10% of a pool of heat-inactivated adult human male AB⁺ serum.

Radioactive labelling technique

Immediately after obtaining the cartilage or after a culture period of 4 days, the samples were labelled for 4 h with an amount of 14.8×10^4 Bq in 10 μl DMEM, $\text{Na}_2^{35}\text{SO}_4$ (Du Pont NEX-041-H, carrier free in 200 μl culture medium). After labelling, the cartilage samples were rinsed three times (45 min) in 1.5 ml medium and washed in 0.5 ml ice-cold phosphate-buffered saline (PBS) [24]. Subsequently, the samples were embedded in an aqueous solution of 8% gelatin white (Sigma), frozen in liquid nitrogen and kept at -80°C until sectioning [31].

Preparation of cryostat sections

Sections (7 μm thick) of fresh frozen cartilage were cut in the sagittal plane on a motor-driven Bright

TABLE I
Details of donors included in the study

Donor no.	Age	Sex	OA grade
1	59	M	4
2	72	M	4
3	70	F	4
4	73	F	4
5	77	M	4
6	70	M	8
7	79	M	8
8	76	M	8
9	75	M	> 8
10	77	M	> 8

From each donor, samples from macroscopically normal regions or pathological looking 'osteoarthritic' regions were taken. Cartilage samples from 'osteoarthritic' regions were classified according to slightly modified Mankin criteria [4, 24]: mild fraying of the articular surface without the presence of cell clones (4 on the Mankin scale); advanced fibrillation of the cartilage surface, characterized by deep clefts and the presence of cell clones (8 on the Mankin scale); extreme fibrillation on the cartilage, the upper cartilage layer is almost totally lost and chondrocytes are only found in cell clusters (> 8 on the Mankin scale).

cryostat fitted with a tungsten carbide-tipped knife at a cabinet temperature of -25°C [32, 33]. Serial sections for autoradiography and immunohistochemistry were collected on poly-L-lysine-coated slides. Sections were kept over silica gel and stored at -25°C until further use.

Autoradiographic localization of incorporated label

Cryostat sections were air dried for 30 min, fixed for 10 min in 4% formalin dissolved in water and subsequently washed three times (5 min) with distilled water. Sections were air dried overnight and subsequently covered with a photographic emulsion (K5, Ilford); the emulsion was diluted 1:1 with 6% gelatin and heated to 45°C . After exposure for 7–14 days, depending on the autoradiographic blackening of normal cartilage, the autoradiographs were developed [34]. For every experiment, i.e. each donor, a normal cartilage sample was analysed together with a pathological sample. The autoradiographic staining was developed depending on the autoradiographic blackening in the normal samples. The normal samples showed minor variation in [^{35}S]sulphate uptake (data not shown).

Immunohistochemistry

Cryostat sections were fixed for 10 min in 4% (w/v) paraformaldehyde (Sigma), dissolved in PBS (pH 7.4) and subsequently washed in PBS containing 10% (w/v) bovine serum albumin (BSA; Sigma, St Louis, MO, USA), 10% (w/v) gelatin and 0.3% (w/v) glycine (Pharmacia, Uppsala, Sweden). Sections were incubated for 30 min at 37°C in a solution of 2 mg/ml testicular hyaluronidase (Sigma) dissolved in PBS to obtain a comparable penetration of antibody into the matrix of normal and OA cartilage. Subsequently, sections were rinsed in PBS and finally treated with 10% (w/v) fetal calf serum and 1% (w/v) BSA in PBS for 30 min to block aspecific binding. Thereafter, sections were incubated for 24 h at 4°C with mouse monoclonal antibody directed against the extracellular α subunit of human IGF-1 type 1 receptor [35, 36] in concentrations of 5 $\mu\text{g}/\text{ml}$ in PBS containing 1% BSA (Oncogene Science, Manhasset, NY, USA). After incubation with the primary antibody, the slides were washed three times in PBS. Sections were incubated with biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, USA) for 2 h in a dilution of 1:500 in PBS containing 1% BSA and 1% normal human serum, and biotin was demonstrated by exposing sections to streptavidin–fluorescein isothiocyanate (FITC) (Boehringer) in a concentration of 40 $\mu\text{g}/\text{ml}$ in PBS. Subsequently, sections were mounted in Vectashield mounting medium (Brunschiwig Chemie) to avoid fading of fluorescence. As a control for α IGF-1 receptor antibody staining, sections were either incubated with mouse IgG or stained in the absence of primary antibody. Sections were kept at -25°C until evaluation with the use of the CLSM.

Fluorescence CLSM and image analysis

The CLSM collects images that are almost free of out-of-focus signals and the system allows optical sectioning of the specimen [37–39]. Immunostained sections were examined with a Leica CLSM attached to a Leica Fluovert microscope using the 488 nm line of an Argon Krypton laser fitted with a 510 nm dichroic mirror and a 530 nm band pass filter, as described in an earlier study [40]. In brief, immunolabelled cryostat sections were subjected to optical serial sectioning with intervals of 0.5 μm in the Z direction.

Quantification of immunofluorescence in the confocal images was performed by area measurement as described previously [40, 41]. Briefly, a window was set over the chondrocyte periphery and over the internal part of the chondrocytes. The distance between the outer rims of the window was kept at $\sim 0.2 \mu\text{m}$. In normal cartilage, the superficial, middle and deeper zones were easily identified ([25]; Fig. 1). For every normal cartilage sample, fluorescence intensity was measured in chondrocytes located in the surface, middle and deeper zones in each of two serial sections. In each cartilage zone, the fluorescence intensity was measured in the chondrocytes that were randomly selected with the use of a microscope ocular with a measuring grid. To assess the contribution of autofluorescence and aspecific binding, fluorescence signals were measured in sections stained with mouse IgG1. For every cartilage sample in each zone, the fluorescence intensity measurement of 10 chondrocytes in two cryostat sections was averaged. For each cartilage sample, the highest average value in a particular zone was set at 100%. The fluorescence signals in the other zones were expressed as a percentage of this value. Data represent the mean \pm S.E.M. of 10 different donors. Statistical evaluation of the experiments was performed with the Wilcoxon rank test. A *P* value of $< 5\%$ was considered to be significant.

RESULTS

Normal articular cartilage

Sample characterization. Figure 1A shows a representative example of articular cartilage defined as normal cartilage. The cartilage samples from macroscopically 'normal' looking cartilage exhibited a smooth intact articular surface, and the superficial, middle and deeper cartilage zones could easily be identified [25]. As characteristics for the various cartilage zones, we used the criteria that chondrocytes in the surface zones were relatively small and flat, their long axis oriented parallel to the surface, whereas chondrocytes in the middle zone were larger, rounded cells existing as single entities randomly distributed in the articular cartilage matrix and chondrocytes in the deeper zones had the largest size, usually occurring in groups. Safranin O staining of the normal cartilage showed intense staining of the middle and deeper zones of the cartilage, whereas the surface zone was devoid of staining (Table II).

Autoradiographic localization of [^{35}S]sulphate incor-

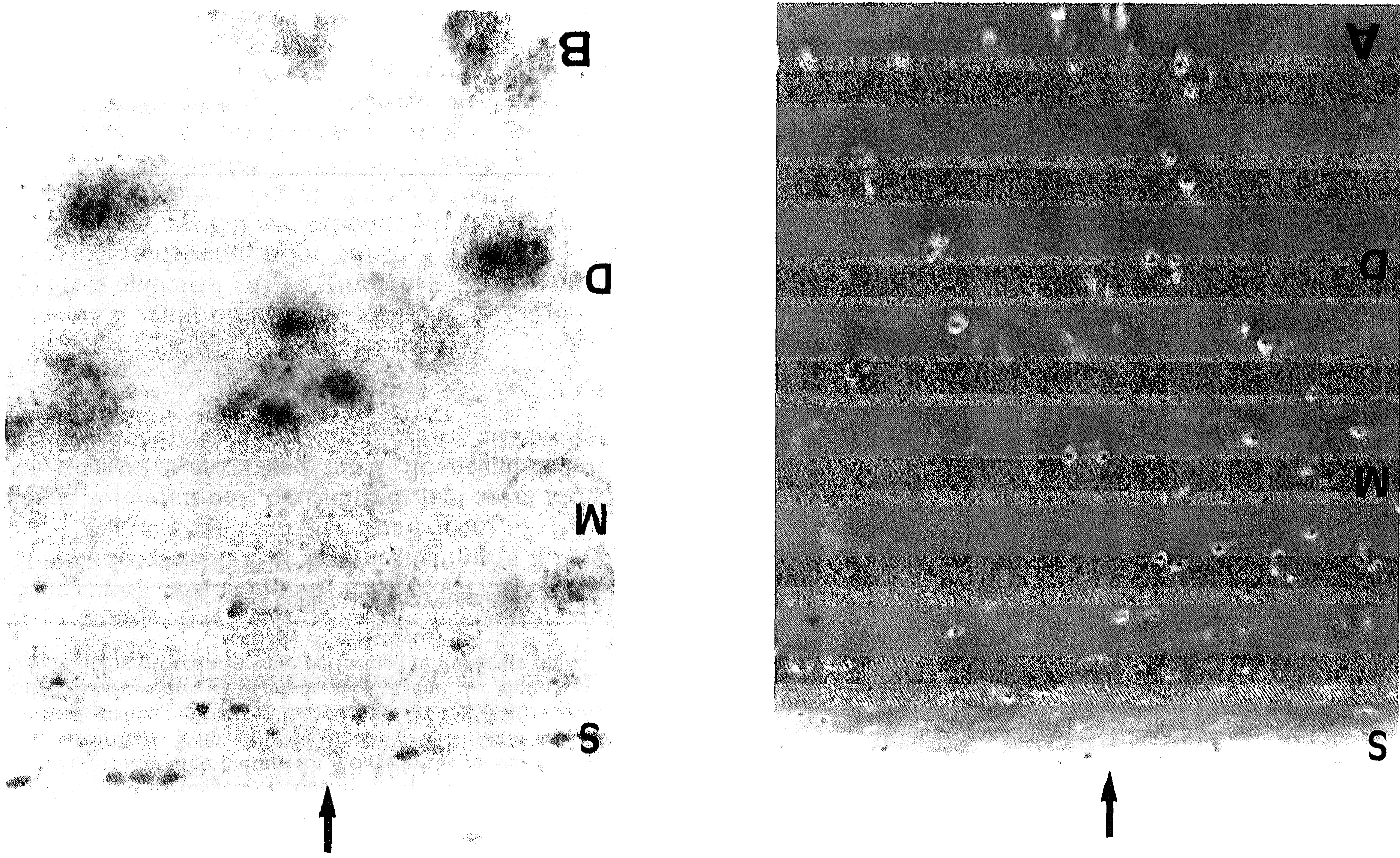


FIG. 1.—Human normal cartilage stained with Safranin O and Fast Green (A), zonal distribution of *in situ* autoradiographic staining of incorporated $[^{35}\text{S}]$ sulphate (B) and digital images of IGF-1 receptor immunostaining (C). For *in situ* autoradiography, the cartilage was labelled with $[^{35}\text{S}]$ sulphate. For IGF-1 receptor localization, cryostat sections were stained immunohistochemically and analysed with CLSM. The representative example of normal human articular cartilage obtained from macroscopically 'normal' looking cartilage regions exhibited a smooth intact articular surface and the surface zone (S), middle zone (M) and deeper cartilage zones (D) could be easily identified (magnification $20\times$). Chondrocytes in the surface zone showed only low levels of $[^{35}\text{S}]$ sulphate incorporation, whereas chondrocytes in the middle and deeper zones of the cartilage displayed distinct amounts of incorporated label (B) (magnification $20\times$). IGF-1 receptor immunostaining of chondrocytes in the surface zone (S) was similar to background fluorescence levels, whereas chondrocytes in the middle (M) and deeper zone (D) showed clear IGF-1 receptor immunoreactivity (C). The uppermost part of the cartilage is indicated by an arrowhead. Bars = $8.5\ \mu\text{m}$.

immunostaining pattern of normal cartilage closely corresponded with the autoradiographic localization of incorporated $[^{35}\text{S}]$ sulphate in normal cartilage. Immunostaining with the $\alpha\text{IR-3}$ monoclonal antibody against the IGF-1 receptor resulted in a clear staining of chondrocytes in the middle and deeper zones (Fig. 1C, Table II). Staining of chondrocytes in the surface zone was similar to background fluorescence

portion. Figure 1B shows the autoradiographic staining of incorporated $[^{35}\text{S}]$ sulphate in normal cartilage. Chondrocytes in the surface zone show only low levels of sulphate incorporation, whereas chondrocytes in the middle and deeper zones of the cartilage display distinct amounts of incorporated label (Table II).

IGF-1 receptor staining. The IGF-1 receptor

TABLE II

Chondrocyte PG synthesis, chondrocyte IGF-1 receptor immunoreactivity and cartilage PG content in human normal cartilage or normal cartilage after culture for 4 days in the presence of serum as well as cartilage from different grades of OA. Data in normal or cultured cartilage represent the mean of 10 donors: five donors of mild OA, three donors of moderate OA and two donors of severe OA. Staining procedures were performed in duplicate on at least 10 sections of each donor

	PG content	PG synthesis	IGF-1 receptor
Normal			
Surface	-	-	-
Middle	++	+	++
Deep	++	++	++
Normal after culture			
Surface	-	++	++
Middle	++	++	++
Deep	++	++	++
OA (4)			
Upper	-	++	-
Deeper	++	++	++
OA (8)			
Upper	-	++	+++
Deeper	++	+++	+++
OA (>8)			
Deeper	++	±	-

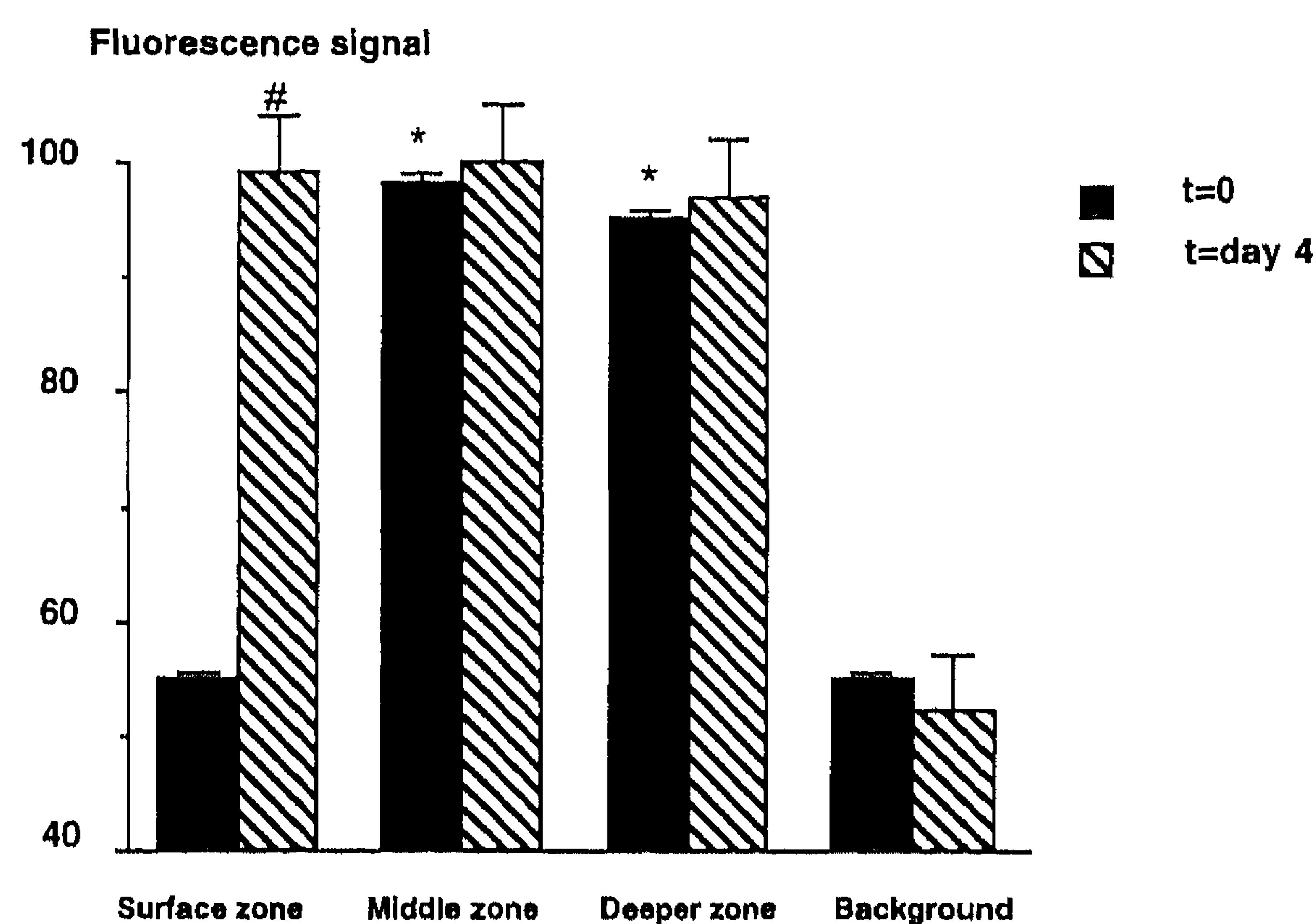


FIG. 2.—Quantification of the fluorescence signal expressed as a percentage in normal human cartilage after incubation with monoclonal anti-IGF-1 receptor antibody. The fluorescence signal was examined in cartilage immediately after isolation of the cartilage or after culture for 4 days in the presence of 10% serum. The fluorescence intensity of each zone was measured in 10 chondrocytes in each of two serial sections. For every normal cartilage sample, fluorescence intensity was measured in chondrocytes located in the surface, middle and deeper zones in each of two serial sections. In each cartilage zone, the fluorescence intensity was measured in the chondrocytes that were randomly selected with the use of a microscope ocular with a measuring grid. To assess the contribution of autofluorescence and aspecific binding, fluorescence signals were measured in sections stained with mouse IgG1. For every cartilage sample in each zone, the fluorescence intensity measurement of 10 chondrocytes in two cryostat sections was averaged. For each cartilage sample, the highest average value in a particular zone was set at 100%. The fluorescence signals in the other zones were expressed as a percentage of this value. Data represent the mean \pm S.E.M. of 10 different donors. Statistical evaluation of the experiments was performed with the Wilcoxon rank test. A P value of $<5\%$ was considered to be significant. *Mean value is significantly different from background fluorescence ($P < 0.05$) as determined by Wilcoxon rank test. #Mean value is significantly different from fluorescence at $t = 0$ in the surface zone.

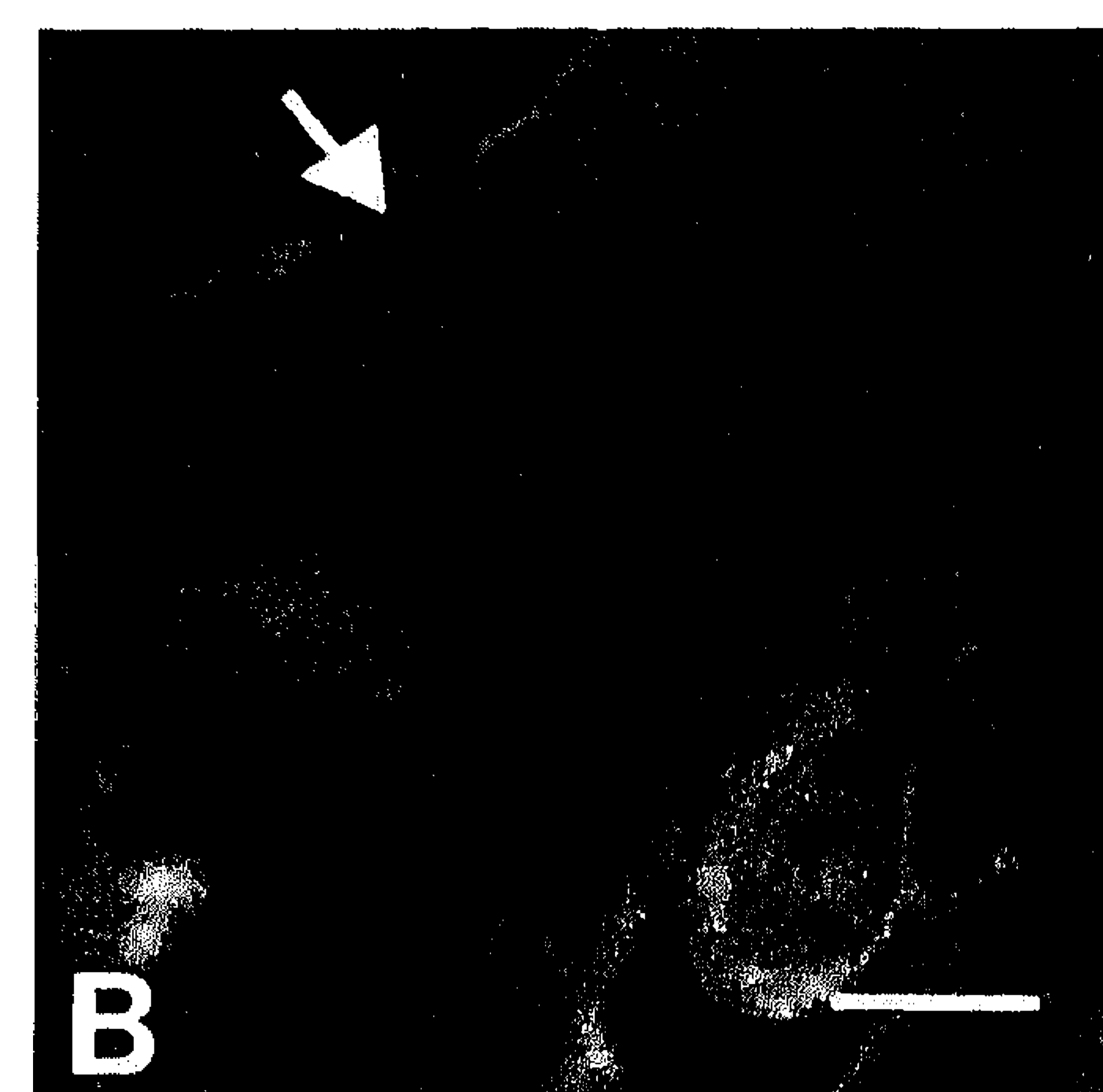
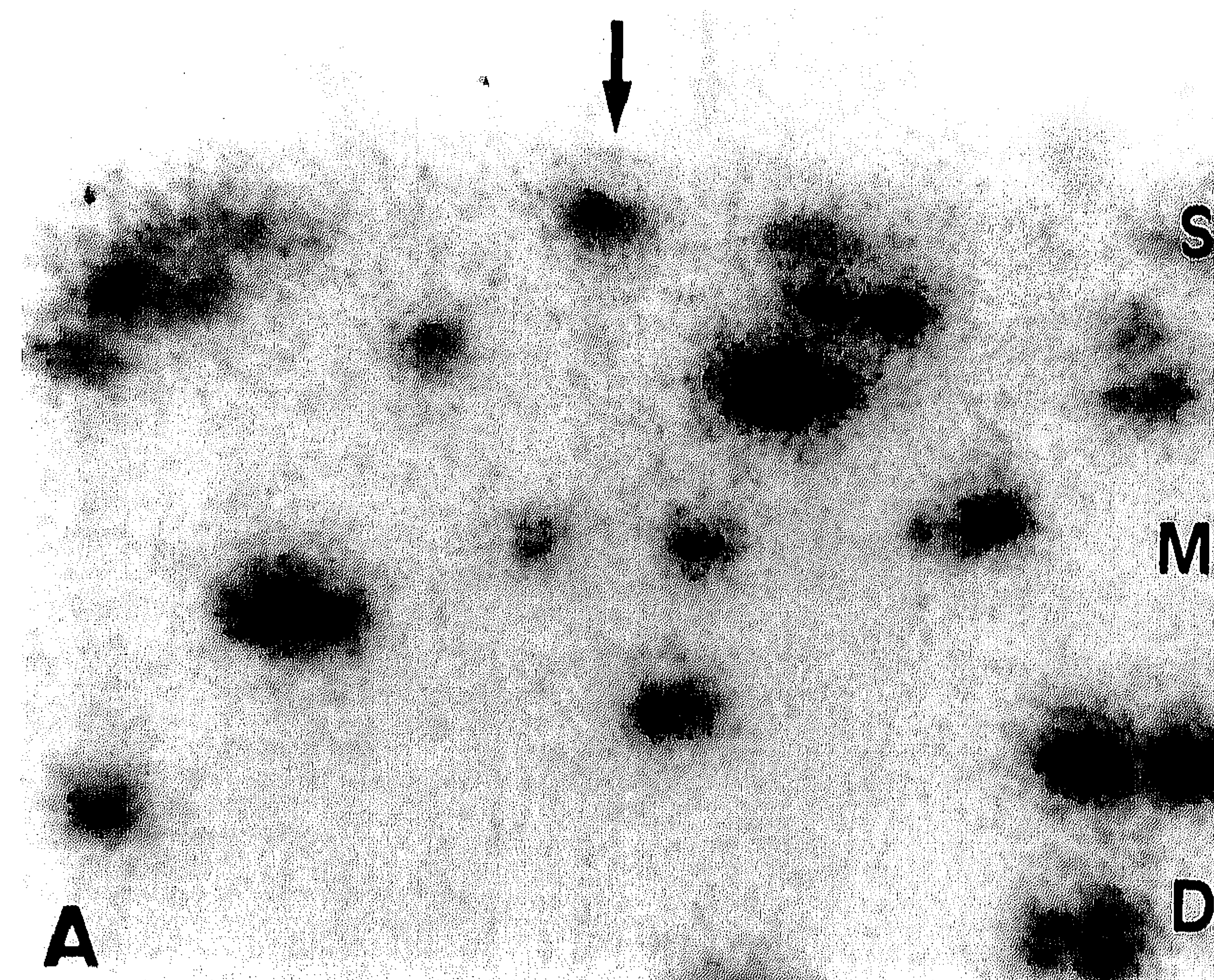


FIG. 3.—Upregulation of the *in situ* autoradiographic staining of the incorporation of [35 S]sulphate (A) (magnification $20\times$) and IGF-1 receptor immunostaining (B) (bars = $8.5\ \mu\text{m}$) in the surface zone of human normal cartilage cultured for 4 days in 10% serum. Surface zone (S), middle zone (M) and deeper zone (D) are shown; the uppermost part of the cartilage is indicated by an arrowhead. For details, see Fig. 2.

levels. Control sections stained either with mouse IgG1 or in the absence of primary antibody showed a faint level of background staining.

Quantification of the immunofluorescence intensity of chondrocytes located in the different cartilage zones is represented in Fig. 2. The fluorescence intensity was determined in the middle optical section of chondrocytes in the surface, middle and deeper zones. The fluorescence intensity of chondrocytes located in the middle or deeper zones of the cartilage was significantly higher than that of chondrocytes in the surface zone, while the intensity of chondrocytes in the surface zone was similar to background intensity. The background fluorescence intensity was at a similar level in all cartilage layers with no significant variation between the independent experiments.

Normal articular cartilage after culture

After culture of normal cartilage for 4 days in the presence of 10% serum, a pronounced significant increase in [35 S]sulphate incorporation was observed in chondrocytes in the superficial zone (Fig. 3A). [35 S]Sulphate incorporation in chondrocytes of the

middle and deeper zones was slightly increased (Table II).

These observations coincide with our findings that the immunostaining of the IGF-1 receptor was also enhanced in chondrocytes in the surface zone (Fig. 3B, Table II). Quantification of the fluorescence intensity revealed a significant increase in IGF-1 receptor immunostaining in chondrocytes in the surface zone as compared with the fluorescence intensity in chondrocytes in the surface zone immediately after isolation of the cartilage (Fig. 2).

OA cartilage

Sample characterization. Cartilage specimens were obtained post mortem from donors without known clinical OA history. The samples were taken from damaged regions with an 'osteoarthritic' appearance and this yielded a wide range of OA grades upon microscopic analysis. A summary is shown in Table I, demonstrating that we collected samples with severe OA ($n = 2$), moderate OA ($n = 3$), but mainly specimens with mild pathology ($n = 5$). Zonal variation in chondrocyte appearance, as found in normal cartilage, was no longer present in OA cartilage. Analysis of OA cartilage was performed in regions classified as upper and deeper layers. The characteristic Safranin O staining of the defined OA categories is shown in Fig. 4. A reduction of Safranin O staining in the upper layer of the cartilage was found in all samples (Table II). Since the severity of OA can vary at various sites of one specimen, special care was taken to analyse the chondrocyte PG synthesis and chondrocyte IGF-1 receptor staining in one area. Thereafter, the samples were divided into two neighbouring specimens that were used for either autoradiography or receptor staining.

Autoradiographic localization of [35 S]sulphate incorporation

The autoradiographic localization of incorporated [35 S]sulphate showed distinct patterns in the three OA categories (Table II). In the cartilage categorized as relatively mild OA, autoradiographic localization did not show the zonal variation as found in normal cartilage. The mild OA cartilage appeared to be uniformly stained. Chondrocytes in the upper and deeper layer of the cartilage exhibited distinct amounts of incorporated [35 S]sulphate (Fig. 5A). The same staining pattern was evident in more severe OA cartilage lesions. The cell clones located in the upper cartilage layer displayed distinct amounts of autoradiographic staining and the chondrocytes in the deeper layers showed high levels of staining (Fig. 5C). However, OA cartilage categorized as extremely severe demonstrated only very low levels of autoradiographic staining (Fig. 5E). The upper layer of the cartilage could no longer be distinguished and chondrocytes in the deeper layer showed only faint [35 S]sulphate incorporation.

IGF-1 receptor staining. IGF-1 receptor immunostaining of cartilage from the three OA categories

showed a diverse staining pattern between the categories (Table II). Relatively mild OA cartilage showed zonal variation in IGF-1 receptor immunoreactivity. Chondrocytes in the upper layer of the cartilage displayed immunoreactivity similar to background staining, whereas the staining in chondrocytes in the deeper layer was obviously high (Fig. 5B). In more severe OA cartilage, the zonal variation was lost; chondrocytes in both the upper and deeper layer exhibited a prominent intense IGF-1 receptor staining (Fig. 5D). In the extreme OA cartilage samples, when the upper layer had disappeared, the immunostaining was indistinguishable from background fluorescence throughout the whole cartilage sample (Fig. 5F).

DISCUSSION

Chondrocyte biosynthetic function plays a key role in maintaining the integrity of the articular cartilage matrix [38]. IGF-1 is the most important anabolic factor, regulating the chondrocyte PG synthetic rate by binding to the type 1 IGF-1 receptor on the chondrocyte membrane [5-18]. Moreover, articular cartilage chondrocytes located in the various cartilage zones exhibit heterogeneity in their metabolic activity [19-22]. We investigated whether chondrocyte PG synthetic activity, chondrocyte IGF-1 receptor localization and cartilage PG content are correlated in the various cartilage zones of human normal cartilage, and in cartilage known to display a shift in chondrocyte metabolic activity, i.e. cultured cartilage and OA cartilage. Because OA lesions occur focally throughout the cartilage, we analysed chondrocyte activity and chondrocyte IGF-1 receptor localization in the same cartilage samples.

In normal cartilage, chondrocyte PG synthesis, IGF-1 receptor localization and cartilage PG content were at low levels in chondrocytes located in the surface zone, but were particularly high in chondrocytes in the middle and deeper zones. These data might indicate that IGF-1 receptor expression in the different cartilage layers reflects heterogeneity in IGF-1 stimulation and metabolic activity of chondrocytes in these layers. Surface zone chondrocytes have been described to exhibit structural, biochemical and biomechanical changes during culture, but also in conditions of cartilage pathology [43-47]. It remains to be seen whether IGF-1 itself is a regulatory factor in some of these pathological events. After culture of the normal cartilage, high levels of both chondrocyte PG synthesis and chondrocyte IGF-1 receptor localization were still found, but the distinct zonal distribution of both chondrocyte synthetic activity and IGF-1 receptor localization disappeared. The shift in the distribution of chondrocyte metabolic activity has been reported earlier in bovine as well as human cartilage explant cultures [20, 24, 48]. In the present study, we have shown that this shift in chondrocyte synthetic activity is correlated with the amount of chondrocyte IGF-1 receptor immunostaining. Therefore, IGF-1 stimulation may contribute to upregulation of the

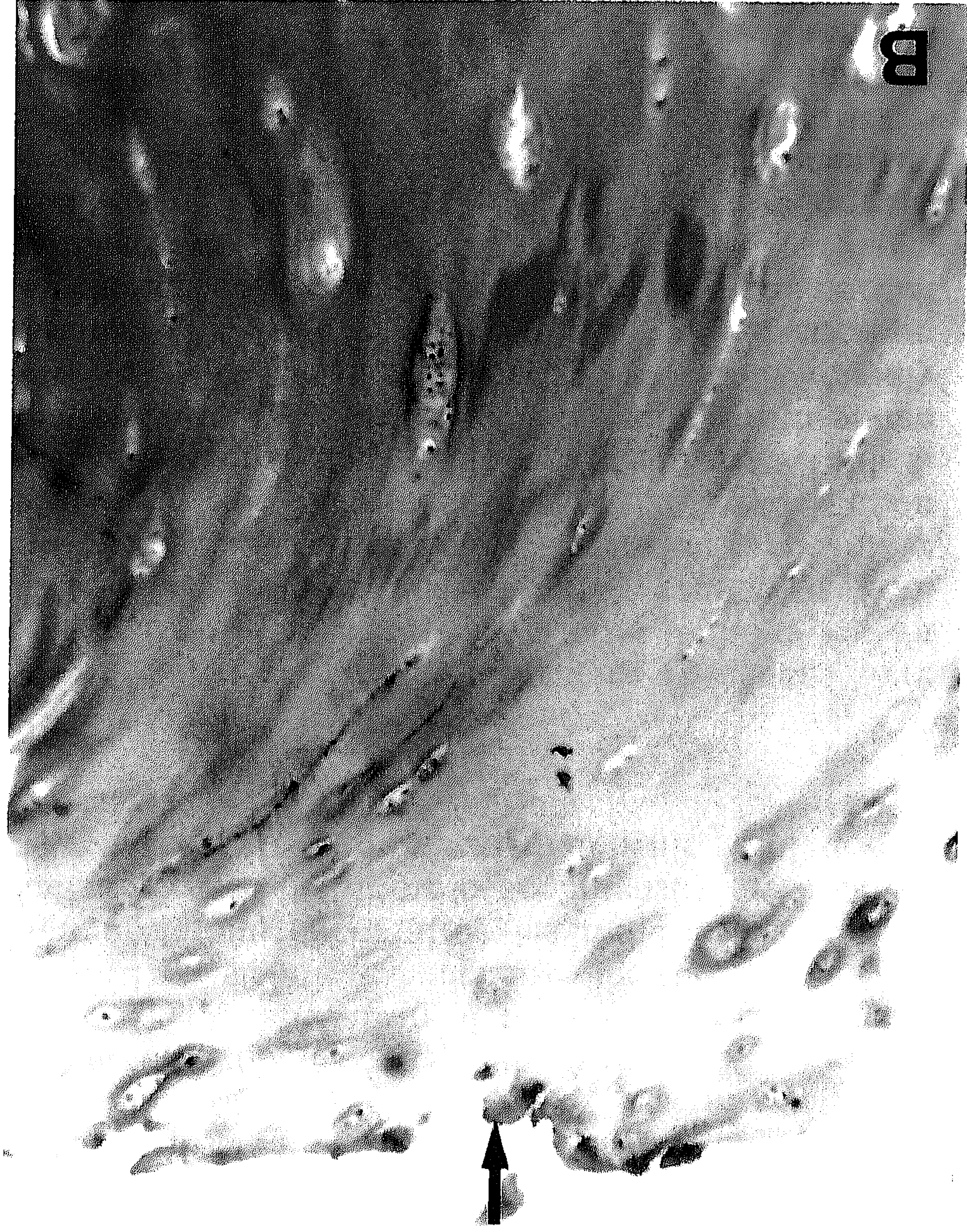


FIG. 4A, B and C (legend opposite).

FIG. 4.—Characteristics of cartilage obtained from damaged cartilage regions with an 'osteoarthritic' appearance stained with Safranin and Fast Green. The cartilage samples were classified according to slightly modified Mankin criteria [4, 24]. Mild fraying of the articular surface without the presence of cell clones is defined as mild OA (4 on Mankin scale) (A). Advanced fibrillation of the cartilage surface, characterized by deep clefts and the presence of cell clones, is classified as moderate OA (8 on Mankin scale) (B). Extreme fibrillation of the cartilage, the upper layer is almost totally lost and chondrocytes are only found in clusters, is defined as severe OA (>8 on Mankin scale) (C). All OA samples showed reduction of Safranin O staining in the upper layer of the cartilage. Zonal variation in chondrocyte appearance as found in normal cartilage was not present in OA cartilage. Therefore, the upper and deeper layer were defined as <150 μm from the articular surface and >150 μm from the articular surface, respectively (magnification 20 \times). The uppermost part of the cartilage is identified with an arrowhead.

biosynthetic activity of resting surface zone chondrocytes during culture.

In earlier studies that examined the role of IGF-1 in the regulation of OA cartilage metabolism, cartilage samples were taken from patients undergoing knee arthroplasty, representing moderate to severe OA cartilage [28, 29]. Our OA samples were obtained post mortem from donors without known clinical OA history, ranging from very mild OA lesions to moderate and extremely severe OA pathology. The very mild OA lesions analysed in the present study are of particular interest; they display a specific pattern that may occur in the very beginning of OA. In mild OA cartilage, the synthetic activity of chondrocytes in the upper cartilage layer was markedly high: the chondrocyte PG synthetic rate was equally distributed over the cartilage specimen. These findings are in line with observations in early experimental OA [49]. The particularly high levels of synthetic activity in the upper cartilage layer indicate that the demand for matrix repair is highest in this layer. Remarkably, in the mild OA cartilage, chondrocytes in the upper layer displayed low levels of IGF-1 receptor immunostaining, whereas chondrocytes in the middle and deeper layers displayed pronounced levels of IGF-1 receptor immunostaining. These results reveal that the role of IGF-1 in the regulation of chondrocyte PG synthesis in the upper cartilage zone in early OA may be of minor importance. Of interest, cartilage from OA lesions has been proven to be much more sensitive to stimulation with transforming growth factor β (TGF β) than normal cartilage [50]. In addition to these *in vitro* findings, we have recently shown that repeated intra-articular injections of TGF β in the murine knee joint caused marked upregulation of chondrocyte PG synthesis, a significant rise in cartilage PG content and a clear development of osteophytes [51, 52]. This situation closely resembles the hypertrophic phase in early experimental OA, indicating that TGF β plays an important role in processes during early OA [53].

Furthermore, in moderate OA cartilage, both chondrocyte synthetic activity and chondrocyte IGF-1 receptor immunostaining were at high levels. The high chondrocyte synthetic activity is known not to reflect a high cartilage PG content in this type of cartilage. This discrepancy in the quality of the cartilage and chondrocyte synthetic activity implies that during this phase of OA the chondrocytes synthesize aberrant PG subtypes causing a less stable articular cartilage matrix. Regarding the severe OA cartilage, our data show that both chondrocyte metabolism and IGF-1 receptor

localization are at low levels. This points to an end stage of OA in which an attempt at increased matrix replacement no longer occurs. At later stages of OA, focal loss of cartilage, enhanced expression of proteolytic enzymes and an imbalance with natural enzyme inhibitors have been described [54, 55].

In addition, in a previous study, we have examined chondrocyte synthetic activity and chondrocyte IGF-1 receptor immunostaining in experimental inflammatory arthritis [52]. In contrast to the high chondrocyte metabolic activity in OA cartilage, chondrocyte PG synthesis is markedly inhibited shortly after the induction of joint inflammation. The cartilage had lost its capacity to respond to IGF-1 stimulation, whereas the amounts of IGF-1 receptors were at normal levels [11, 12]. In comparison with the data found in mild OA cartilage, correlation of chondrocyte metabolic activity and chondrocyte IGF-1 receptor immunostaining was disturbed, suggesting inadequate or overruled signalling.

The methods used in the present study enabled *in situ* comparison of chondrocyte PG synthesis, IGF-1 receptor immunostaining and the cartilage PG content. The data obtained are still semiquantitative and do not allow concrete statistical correlations. Moreover, IGF-1 receptor immunostaining may at best give an indication of IGF-1 receptor expression. However, quantitative assays will need isolation of the chondrocytes; analysis of the whole chondrocyte population will lead to loss of information about chondrocytes located in the different cartilage zones. Therefore, the experiments performed in the present study are of advantage giving information on *in situ* PG synthesis, PG content and IGF-1 receptor immunostaining.

In summary, in normal cartilage, both the amount of chondrocyte PG synthesis and chondrocyte IGF-1 receptor localization are at low levels in surface zone chondrocytes, and high in middle and deeper zone chondrocytes. This indicates that IGF-1 displays cellular heterogeneity in chondrocyte stimulation in the various cartilage zones. After culture, the shift in chondrocyte PG synthesis is correlated with an increased IGF-1 receptor immunofluorescence in the surface zone. Therefore, IGF-1 stimulation contributes to upregulation of resting surface zone cells during culture. In mild OA, the particularly high levels of chondrocyte synthetic activity in the upper cartilage layer did not correlate with amounts of IGF-1 receptor immunostaining, indicating that other factors play a role in the chondrocyte regulation in this phase of OA. The pronounced high chondrocyte PG synthetic

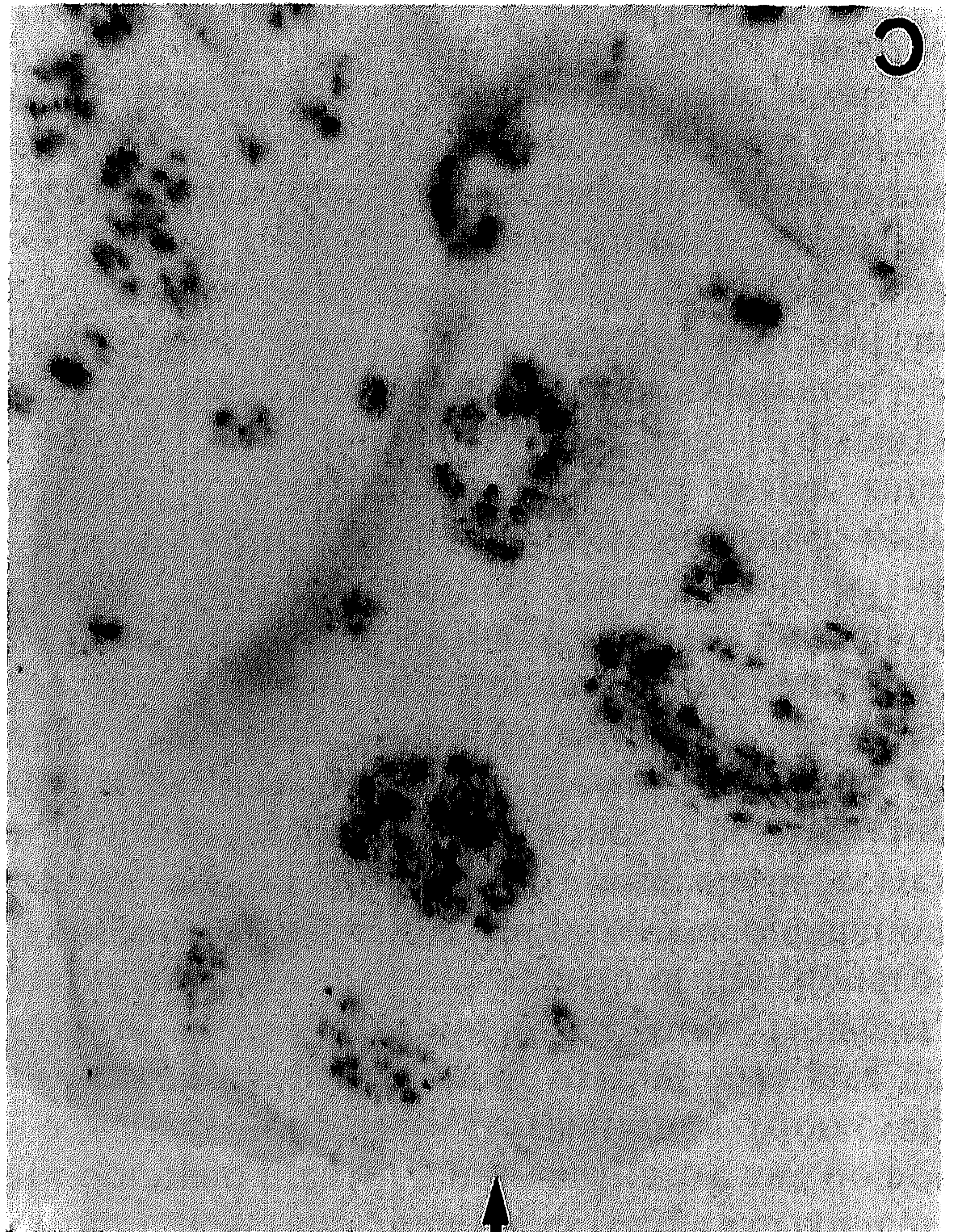
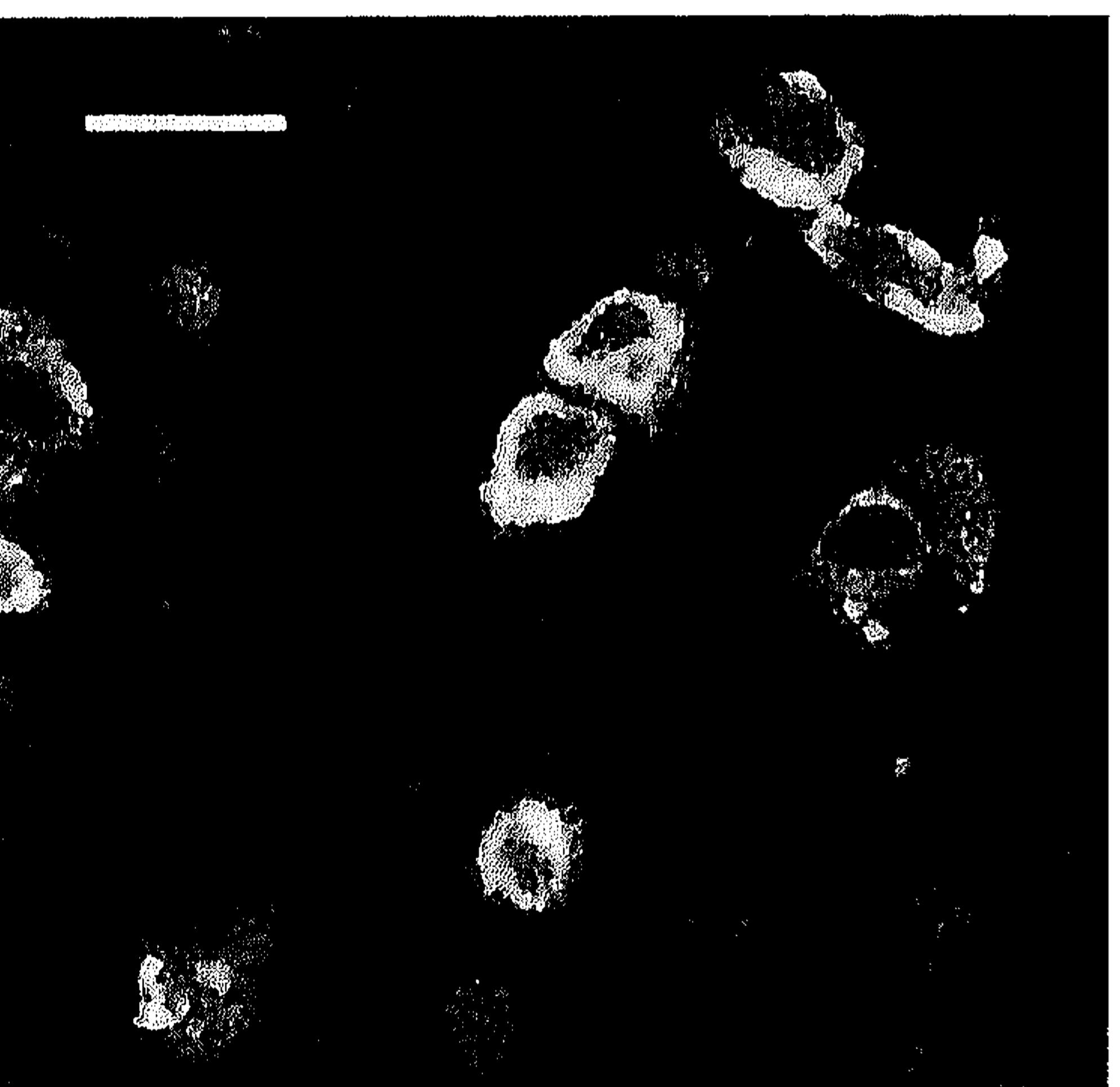
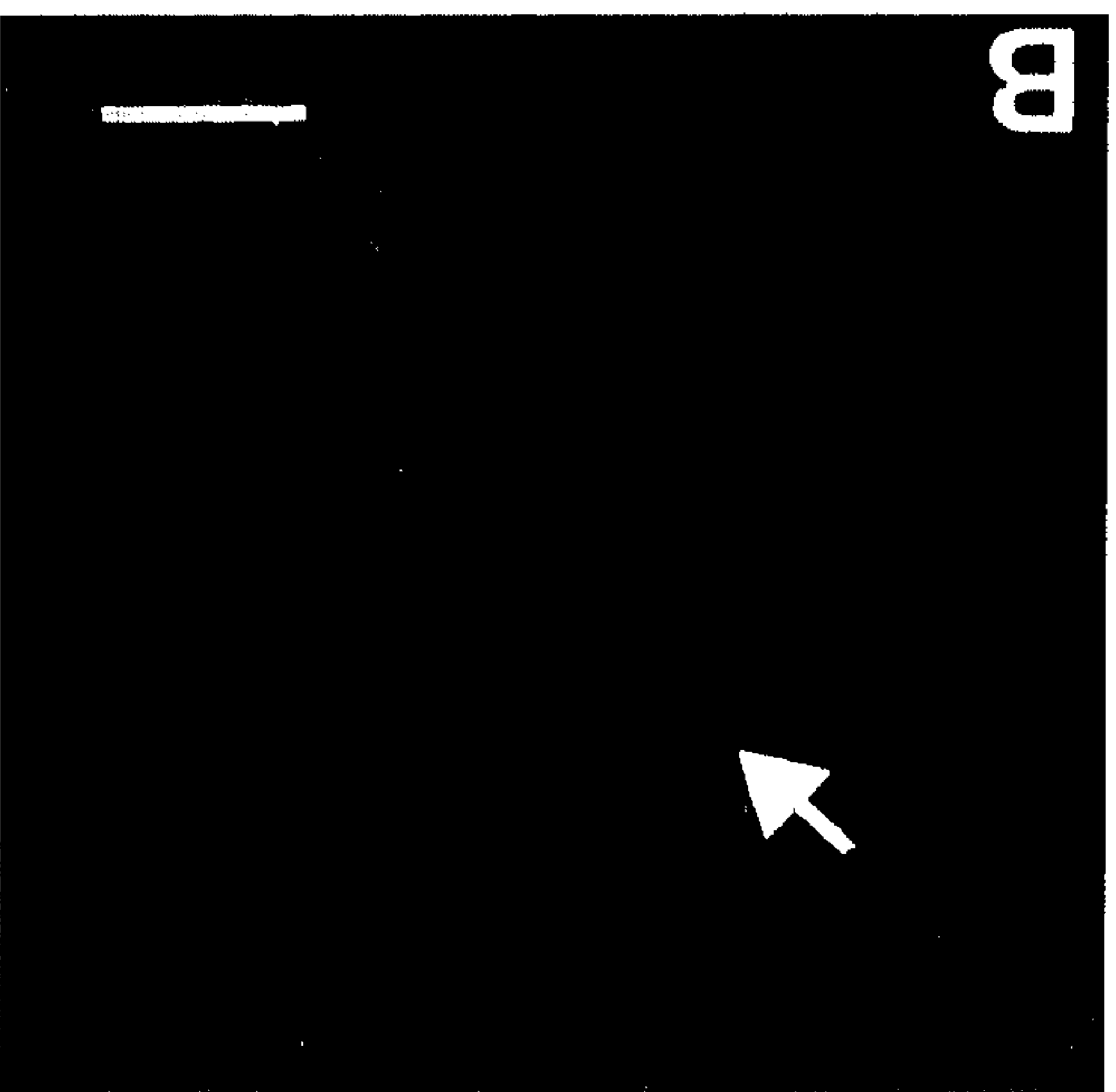
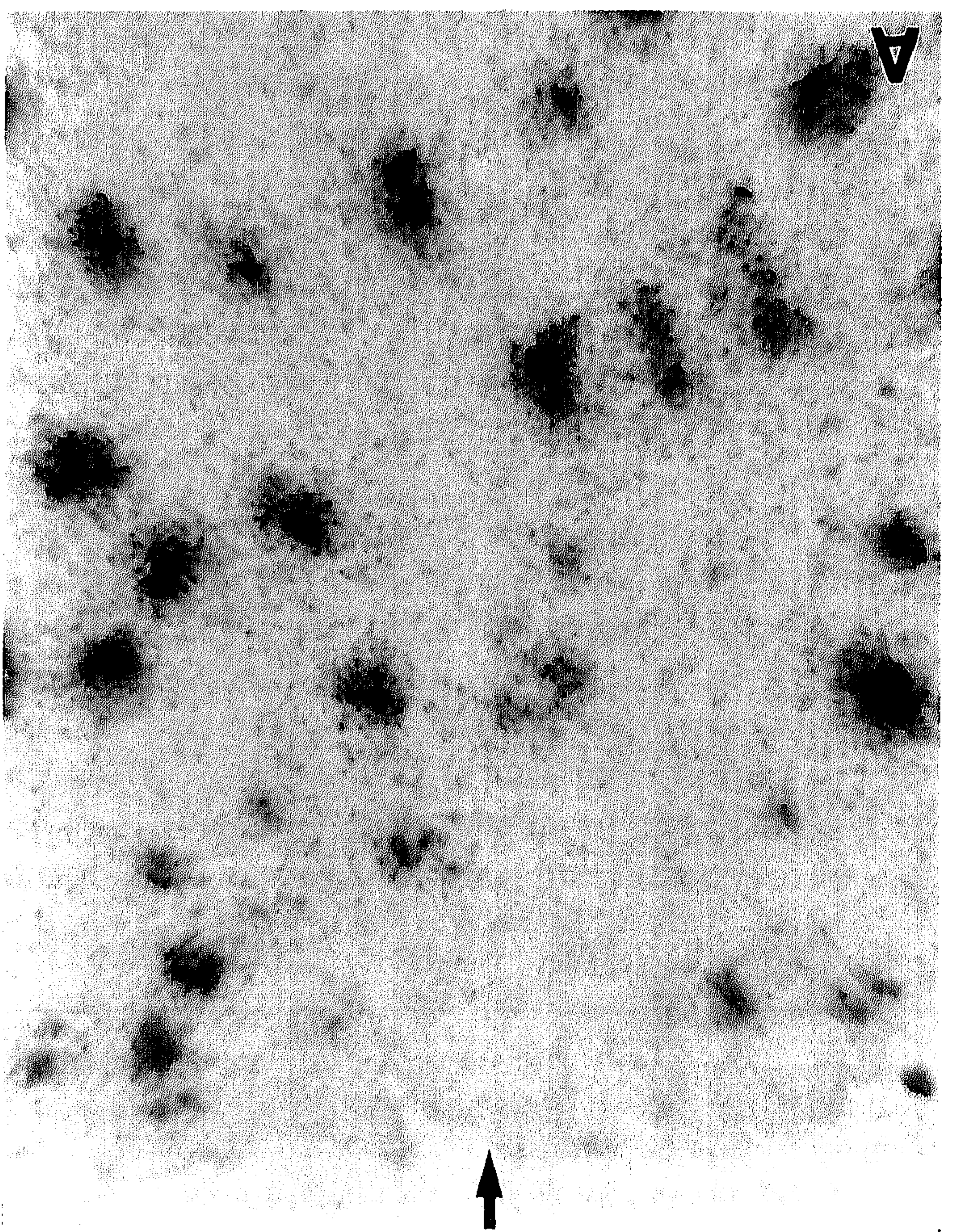


FIG. 5. A-D.

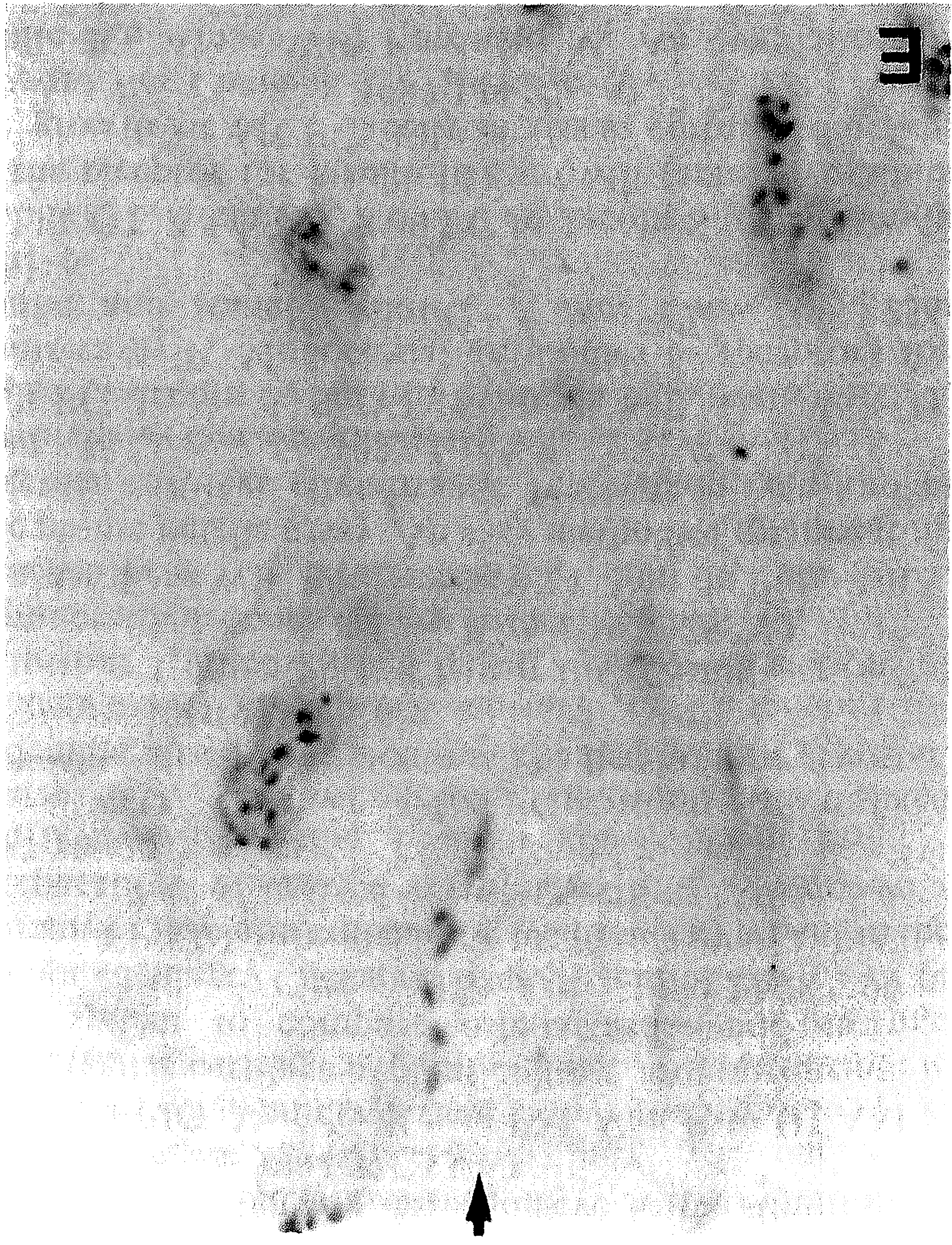


FIG. 5. A-D. Distribution of *in situ* autoradiographic localization of incorporated [³⁵S]sulphate (A, C, E) (magnification 20×) and IGF-1 receptor staining (B, D, F) (bars = 8.5 μm) in the three defined OA categories: mild OA (A, B), moderate OA (C, D) and severe OA (E, F). The uppermost part of the cartilage is indicated by an arrowhead. Mild OA cartilage shows no zonal variation in chondrocyte PG synthesis, but appears to be uniformly stained (A). Chondrocytes in the upper layer of mild OA cartilage display IGF-1 immunoreactivity similar to background staining, whereas staining of chondrocytes in the deeper layer is obviously high (B). Moderate OA cartilage shows an apparent high PG synthesis both in the upper and deeper cartilage layer (C). Chondrocytes in moderate OA cartilage show an intense IGF-1 receptor staining in the upper and deeper layer (D). Severe OA cartilage exhibits only very low levels of chondrocyte PG synthesis (E). Chondrocytes in severe OA cartilage display immunostaining indistinguishable from background fluorescence throughout the whole cartilage sample (F).

activity and chondrocyte IGF-1 receptor staining in moderate OA cartilage may represent an active attempt at repair, whereas the low levels of chondrocyte activity and IGF-1 receptor localization in severe OA may be indicative of an end stage of OA.

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