Abstract. This study provides morphometric data on radiation-induced alterations of the facial nerve in female Wistar rats. The facial nerves were explanted 3 to 4 or 7 to 9 months after completion of a fractionated external irradiation of the left side of the neck (5 days per week, 6 weeks, total: 60 Gray). Both facial nerves were investigated in order to identify possible effects of scattered irradiation on them. A total of 54 animals were investigated and 11,193 measurements were obtained. The computer-assisted image analysing system CUE-3 Color Image Analyzer (Olympus, Japan) was used. Facial nerves from untreated animals served as controls. Results: Three to four months after irradiation, the ratio of axon area and total area of the cross-sectioned nerve was shifted towards the axon area. Seven to 9 months after completion of irradiation, both the myelin sheath and the axon were reduced in diameter. The loss of substance affected predominantly the myelin sheath. Peripheral nerves are radiosensitive. Seven to 9 months after irradiation there was an increase of the axon area compared to the total area, both in absolute values and in relative terms. Radiosensitivity of peripheral nerves can be measured morphometrically in suitable animal models. The measurements disclosed temporal and spatial patterns of radiation response of the nerves. These results may partly explain the increased radiosensitivity of the peripheral nervous system observed in the long-term follow-up after completion of radiotherapy of head and neck in humans.

The base of the skull, face and neck, inevitably lie, at least in part, in the treatment field of radiotherapy for head and neck cancer (1, 2). Due to the complex embryology of the head, the cranial and neck nerves are densely interwoven with radiosensitive organs that can give rise to malignancy (3, 4). Therefore, the simultaneous exposure of nerves not affected by the malignant disease to the radiation source must be taken into account in the estimation of putative radiation damage of unaffected tissues (5-8). Furthermore, interactions of different tissues in the irradiation field may contribute to nerve dysfunction (9-12). Fortunately, peripheral nerves are considered to be radioresistant (13-16). Indeed, peripheral nerve wound healing in terms of unimpaired signal conduction of experimental nerve autografts appears to be unaffected by radiation (14). Regeneration of function has also been demonstrated for facial nerve autografts in irradiated patients (15, 16). However, the success rates of facial nerve graft vary considerably and data providing long-term patient outcomes are scarce.

Some studies on the effect of irradiation on peripheral nerves, however, showed certain sequelae after radiotherapy that have to be attributed to the radiation exposure of nerves in the radiation field rather than to the neoplasm (18-26). Damage of cranial nerve is frequently reported in the follow-up for head and neck cancer (8). There are only few studies published on morphological alterations of cranial nerves following irradiation (1, 2, 24-26). To date there are no systematic morphological studies published on the effect of irradiation on the facial nerve, in particular considering an irradiation protocol and total dosage that is used for head and neck cancer in humans. The aim of the present experimental study was to provide experimental data on the effect of irradiation on the facial nerve.
Materials and Methods

Nerves. The facial nerves were explanted from 54 healthy female Wistar rats weighing 220 to 280 g at the beginning of the experiments (source: Charles River, Sulzheim, Germany). Out of these animals, 37 were not irradiated so that their nerves served as a control (68%). Following the completion of the irradiation, 8 out of the 17 irradiated animals were killed at 3 to 4 months (15%) and 9 animals were killed at 7 to 9 months (17%). After supravalvital peritoneal injection of pentobarbital, the animals were perfusion-fixed at room temperature with glutaraldehyde as described in detail elsewhere (27-30). The nerves were excised with a part of the masseter muscle in order to facilitate the orientation of the immersion-fixed samples for preparing cross-sections of the facial nerve. The tissues were then osmosed in phosphate-saccharose, dehydrated in graded ethanol and embedded in epon. Semi-thin cross-sections of the nerves (1 μm) were cut with an ultramicrotome, stretched in a water bath, placed and dried on a slide, and stained with toluidine blue.

Morphometrics. Measurements were performed using a light microscope (Type BH2; Olympus, Japan) supplied with a CCD video camera to digitise the region of interest. A computer-aided image analysis system CUE-3 Color Image Analyzer (Olympus, Japan) was used for morphometric analysis. Continuous interactive control of the morphometric procedure was visualised on a screen. Neither the silhouette of the axons nor the myelin sheaths could be registered automatically by the image analysis software, therefore the shapes of these structures were tracked manually with the cursor on the fixed image following the image processing. Four regions of each single cross-sectioned nerve were measured, each containing 25 nerve fibres, resulting in 100 nerve fibres per cross-section of nerve. A total of 11,193 measurements were performed (non-irradiated nerves: n = 7,767; 3 to 4 months after irradiation: n = 1,531; 7 to 9 months after irradiation: n = 1,895). Both facial nerves were explanted and investigated in the same manner for all animals. In the control group, 4,595 measurements were made on the left nerves and 3,172 measurements on the right nerves. For 7 to 9 months after irradiation, 620 measurements were performed on the left and 842 on the right facial nerves. For 7 to 9 months after irradiation, 842 measurements on the left and 1,053 measurements on the right facial nerves were performed.

Areas and diameters. Different parameters (areas and diameters) of the nerves were evaluated to assess the effect of irradiation on the nerves: the area of a single nerve fibre is given in square microns (termed “microns area”, μm²) and was calculated automatically after manual tracking of the structure shape. Mean values of the diameter of a cross-sectioned nerve fibre are termed “average ferets” (μm). The mean values were calculated based on 8 Martin’s radii. A Martin radius is the distance of the midpoint of an object to the border of the object in defined angles (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°). The area of an axon is the “hole area” (μm²) and the ratio of axon to total area of the fibre defines the “hole ratio” (Figure 1).

Irradiation procedure. Seventeen animals were subjected to a total dosage of 60 from a conventional X-ray therapy source installed in a lead chamber adapted to experimental irradiation of small animals. The equipment is described elsewhere in detail (27-30). Figure 2 shows the irradiation field of the animals. The irradiation experiments approximate the situation of a representative human external head and neck irradiation by means of total dosage (60 Gy) and fractionation (2 Gy/day, 5 times a week), resulting in an irradiation period for each animal lasting 6 weeks. The irradiation was performed in a dose-controlled manner and restricted to the left side of the neck and skull base. The dosimeter was calibrated daily prior to the irradiation procedure. The daily exposure to the radiation source lasted about 90 seconds and was performed under general anaesthesia. Technical details are described in detail elsewhere (27-30). All experiments were approved by the local ethics committee and were performed according to the German animal protection law.

Statistics. The data sets were analysed statistically using SPSS™ (version 12.0; SPSS Inc, Chicago, IL, USA; t-test, Levene-test, Scheffé-test).

Results

Descriptive analysis of fibre parameters

Area of single fibre. A total of 11,193 measurements of single fibres of facial nerves were made. The values ranged from 4.14 μm² to 201.99 μm² (mean: 39.67 μm², standard deviation (SD): 21.04). The distribution of the areas of single fibres approximately followed a Gaussian distribution, with a median area of 35.91 μm².

Surface area of single fibres of irradiated nerves. The calculations were based on 1,462 measurements of single fibres of directly irradiated left facial nerves. The control group constituted 4,595 measurements of left facial nerves without any irradiation or other noxae. The single fibres had a mean surface of 37.73 μm² (SD 18.78) in irradiated animals and 40.22 μm² (SD 21.67) in the control group. The difference in mean surface area between the groups was 2.49 μm² and was statistically significant (p=0.001).

Whether the time interval between completion of irradiation and investigation had a significant impact on the fibre areas was investigated. The values were calculated using unifactorial analysis of variance (Scheffé-test, error probability <5%). Morphometric differences between the values of the control group and irradiated animals were caused by alterations measured in nerves explanted after 7 to 9 months after completion of irradiation. Whereas the single fibre areas from non-irradiated nerves and nerves collected 3 to 4 months after irradiation did not differ significantly, the single fibre areas of animals 7 to 9 months after irradiation were reduced with a mean value of 35.27 μm² (SD 17.54) compared to controls (mean: 40.22 μm²; SD: 21.7) and 3 to 4 months after completion of irradiation (mean: 41.08 μm²; SD: 19.87). The difference between the 3 to 4 months group and controls was statistically non-significant.
Mean diameter of single fibre. Automated measurement of diameter of the single fibres revealed a range from 0.005 μm to 25.48 μm (mean: 7.16 μm).

Axon area. Axon area varied considerably (0.001 μm² to 80.18 μm², n = 11,156 values), with a mean axon area of 14.639 μm² (SD 9.00). The median value (12.78 μm²) was lower than the mean value: 50% of the axon areas fall in the range 7.88 μm² to 19.55 μm². This indicates a non-Gaussian distribution of the axon area.

Comparison of axon area and total area of single fibres. The calculation of the ratio of axon area and total area of a single fibre (‘hole ratio’) revealed values in the range from 0.001 to 0.74 (mean 0.36, SD 0.07). The median of axon/total area ratio of single fibres is 0.36. The small difference between median and mean values demonstrates the Gaussian distribution of measured values.

Area of single fibre (microns area). A total of 1,462 measurements of irradiated facial nerves were compared to 4,595 measurement values of facial nerves from control animals. The mean single fibre area of irradiated animals was 37.73 μm² (SD 18.78) compared to 40.22 μm² (SD 21.69) of untreated controls. The difference between these mean values (2.49 μm²) was statistically significant (p=0.001).

The latency between radiation and morphological effect on the nerves was investigated. The mean value of single fibre areas in nerves of the 7 to 9 months latency-period group were significantly lower compared to those obtained in controls and in the 3- to 4-month latency period group (control group vs. 3- to 4-month latency period group, non-significant differences).

Mean diameter of single fibre. The mean diameter of a single fibre of irradiated animals was 6.95 μm (SD 1.83) compared to 7.21 μm (SD 2.06) in the control group. The difference of 0.26
μm between the values of both groups was statistically significant ($p=0.001$). The detailed evaluation of the mean diameters of single fibres revealed the same correlation as shown for the comparison of fibre areas: animals with a 7- to 9-month latency period had significantly lower values compared to the controls and the 3- to 4-month latency period group ($p<0.001$). A trend towards increased mean diameter values for the 3 to 4 months group compared to controls was found, but proved to be statistically insignificant.

**Axon area.** The axon area was reduced in irradiated animals. However, this reduction was statistically insignificant ($p=0.18$). The mean axon area of irradiated animals was 14.27 μm$^2$ (SD 8.24) compared to the mean axon area in controls (14.64 μm$^2$, SD 9.37).

Whereas the comparison of measurement values of irradiated animals (whole group) and controls revealed no difference, the analysis of sub-groups revealed a significantly reduced axon area in the 7 to 9 months group (compared to controls and 3- to 4-month latency period irradiated animals).

**Ratio of axon/total area of single fibres.** The ratio of axons to the total area (hole ratio) was significantly higher in irradiated animals compared to controls ($t$-test: $p=0.001$). The hole ratio of irradiated animals was 0.37 (SD 0.07). The axon occupied 34.9% (mean value) of the total area of single fibres (SD 7.6) of untreated controls.

The hole ratio was the lowest in controls (0.35; SD 0.08). In the 3- to 4-month latency subgroup, the hole ratio was 0.37, while in the 7- to 9-month latency subgroup it was 0.36 (SD for both subgroups was 0.07).

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**Impact of irradiation scattering effects on fibre measurements**

**Area of single fibre.** A total of 1,964 measurements were obtained from single fibres of the right (shielded) facial nerve of animals irradiated on the left neck and skull base region. These values were compared to 3,172 values obtained from right facial nerves of untreated controls. The mean area of single fibres that were possibly exposed to scattering irradiation was 39.04 μm$^2$ (SD 19.28). Values of the control group were in a similar range (40.17 μm$^2$, SD 22.03). The difference between both mean values was of borderline significance ($p=0.062$).

The investigation of the impact of the time elapsed after completion of radiation on the values revealed that the 3- to 4-month latency period group had significantly lower fibre areas (Scheffé-test).

**Mean diameter of a single fibre.** Single fibres of right facial nerves from irradiated animals had a mean diameter of 7.07 μm (SD 1.88) compared to 7.23 μm (SD 2.15) in controls. This difference was statistically significant ($p=0.005$).

Unifactorial analysis of variance revealed that differences of single fibre diameter between right facial nerves of the control group and the irradiated groups were caused by the values of the 3- to 4-month latency period group. The mean diameter of a single fibre of the control group was 7.23 μm (SD 2.11) compared to 6.96 μm in the 3- to 4-month latency period group (SD 1.81). Diameters of nerve fibres investigated 7 to 9 months following irradiation showed no differences when compared to either group (control or short-term latency).

**Axon area.** The axon area of facial nerves in scattering-irradiation exposed animals was lower (14.47 μm$^2$; SD 8.53) compared to nerves from non-irradiated (14.75 μm$^2$; SD 9.12). However, this difference was not statistically significant ($p=0.27$). In contrast to the result of overall comparison between irradiated and non-irradiated nerves, the unifactorial analysis of variance demonstrated a statistically highly significant time-dependent difference of scattering effects on axon areas in irradiated animals ($p=0.001$). Nerves of animals investigated 3 to 4 months after irradiation showed a significantly reduced axon area (mean: 13.49 μm$^2$; SD 7.7) compared to the control group (mean: 14.75 μm$^2$; SD: 9.17) and the long-term latency group (mean: 15.31 μm$^2$; SD 9.08). Axon areas of nerves of the 7 to 9 months group did not differ from those in controls.

**Axon/total area ratio of single fibre.** The hole ratio of a single fibre did not differ significantly between animals exposed to scattering irradiation and controls. In both groups, the hole ratio of a single fibre was about 35.6% (SD 7.3 and
Comparison between irradiation effects on facial nerves in the irradiation field and those outside the irradiation field (scattering irradiation effects)

Area of single fibre. The mean area of single fibre was 37.73 μm² (SD 218.78) in nerves inside the irradiation field and 39.04 μm² (SD 21.83) in nerves of suspected scattering irradiation exposure. In contrast to these measurements, the mean fibre area of control animals was 40.2 μm². Statistically significant differences were restricted to fibre areas of controls and nerves inside the irradiation field.

The investigation of nerves harvested after 3 to 4 months revealed differences between nerves exposed to scattering irradiation compared to those with direct irradiation exposure or no irradiation exposure. This effect was also demonstrated for the long-term latency group (7-9 months).

Mean diameter of single fibre. The mean diameter of single fibres in control animals was 7.22 μm (SD 2.08 μm), 6.95 μm (SD 1.83 μm) in directly irradiated nerves, and 7.07 μm in nerves with suspected scattering-irradiation effects. Differences in mean diameter of facial nerves inside or outside the direct irradiation field proved to be insignificant.

Analysis of subgroups revealed differences of the diameters in the 3- to 4-month latency period group comparing left and right nerves. There was no difference in mean diameter of single fibres comparing untreated nerves and those from the short-term control of directly exposed nerves. However, diameters of single nerve fibres inside the irradiation field within the 7- to 9-month latency group differed significantly from those of nerves outside the irradiation field and controls.

Axon area. Analysis of variance revealed no significant difference in axon area between the three groups. The mean axon area was 14.68 μm² in the control group (SD 9.29), 14.271 μm² in nerves inside the irradiation field (SD 8.24), and 14.47 μm² in nerves outside the irradiation field (SD 8.53).

The analysis of variance revealed significant differences of measurements, taking into account the latency period following the completion of irradiation exposure. The axon area of nerves outside the irradiation field, explanted after 3 to 4 months, differed significantly from those that were directly exposed to the radiation source and from controls. In contrast, the nerves that were explanted after 7 to 9 months showed significant differences of directly exposed nerves compared to nerves of untreated animals and nerves with probable scattering irradiation.

Ratio of axon/total area of single fibres. For this analysis, the measurements of nerves explanted 3 to 4 months or 7 to 9 months following irradiation were pooled into a single group. The hole ratio of irradiated vs. non-irradiated nerves proved to be highly significant. The hole ratio of nerves inside the irradiation field differed significantly from the values of untreated animals and the scattered irradiation samples (facial nerves of the right side of irradiated animals). The was no difference of hole ratio between control and scattering effects group.

Three to four months following irradiation, the hole ratio of all groups differed significantly. In the control group, the ratio was 35.2% (SD 7.8), inside the irradiation field 37.2% (SD 6.8), and outside the irradiation field 34.5% (SD 7.2). Analysis of the 7 to 9 months group revealed differences between the control and inside the irradiation field (mean: 36.4%; SD 6.8) and outside the irradiation field (mean: 36.5%; SD 7.2). The values of both groups of nerves of irradiated animals with varying exposure to the irradiation source showed no statistically significant differences.

Interpretation of measurements

Area of single fibre. The area of single fibre (microns area) in facial nerves inside the irradiation field 3 to 4 months following completion of irradiation showed no significant difference compared to untreated nerves. However, 7 to 9 months after irradiation, nerve fibre area was reduced significantly compared to nerves of untreated animals ($p<0.05$). Scattering irradiation resulted in a reduction of single fibre area 3 to 4 months after irradiation ($p<0.05$).

Mean diameter of single fibres. The mean diameter of single fibres (average feret) of facial nerves inside the irradiation field showed no significant difference of measurement values compared to controls. However, 7 to 9 months after irradiation, similar to the measurement values of axon area, a reduction of average feret was measured ($p<0.05$). Scattering irradiation-induced reduction of average feret was detected in the 3 to 4 months group only ($p<0.05$) and not detectable after 7 to 9 months.

Axon area. The area of the axons (hole area) of facial nerves exposed directly to the irradiation source that were investigated 3 to 4 months after completion of irradiation showed distinct differences from values obtained from controls (strong tendency for increased diameters in irradiated animals). However, the 7 to 9 months group revealed a significant reduction of axon area ($p<0.05$). The impact of scattering radiation on facial nerves was obvious.
3 to 4 months after irradiation ($p<0.05$). However, after 7 to 9 months, a significant increase of axon area was noted in shielded nerves compared to nerves from non-irradiated animals ($15.31 \, \mu m^2$ vs. $14.68 \, \mu m^2$, $p<0.05$).

**Ratio of axon and total area.** The ratio of axon and total area (hole ratio) was significantly elevated in facial nerves inside the irradiation field compared to controls at the 3 to 4 months interval ($37.2\%$ vs. $35.2\%$, $p<0.05$). In the 7 to 9 months group, this difference declined but was still significant ($36.4\%$ vs. $35.2\%, p<0.05$).

The impact of scattering irradiation was a significant reduction of the ratio in the 3 to 4 months group ($34.5\%$ vs. $35.2\%$, $p<0.05$). The values obtained in nerves investigated after 7 to 9 months showed similar relations compared to nerves inside the radiation field ($36.5\%$ vs. $35.2\%, p<0.05$). Figure 1 shows the alterations of the morphometric parameter in nerves inside and outside the irradiation field, compared to untreated animals.

**Discussion**

This investigation revealed the effect of fractionated irradiation (60 Gy) on facial nerve fibres inside the irradiation field. Four parameters were investigated in nerve fibres 7 to 9 months after completion of the protocol: area of single fibre, mean diameter of single fibre, axon area and axon to total area ratio. The measurements showed significant differences between fibres of irradiated vs. non-irradiated animals. These results indicate a loss of substance of both the myelin sheath and the axon in irradiated nerves. The increase in the axon to total area ratio demonstrates a predominance of myelin sheath in this shrinkage process.

However, no significant difference of area of single fibre, mean diameter of single fibre and axon area were identified in the facial nerves investigated 3 to 4 months after direct exposure to the radiation source. The axon to total area ratio was changed in favour of the axon, indicating the swelling of the axon as a result of irradiation.

The findings obtained in the long-term observation group were in accordance with earlier findings reported by Spiess (25, 26). In a similar experimental setting using rats and based on electron microscopy investigations, Spiess revealed alterations of the axon that were supposed to indicate a reduction of neurofilaments and apoptosis of Schwann cells (25, 26).

Results in this study indicate that: (i) peripheral nerves are subject to chronic nerve damage, (ii) chronic nerve damage follows a chronological sequence and (iii) chronic nerve damage is quantifiable morphometrically.

This study provides evidence for a time sequence of irradiation-induced cranial nerve damage but does not indicate the mechanism of damage. The mode of action of irradiation on nerves is a currently debated issue. Radiation-induced endothelial cell damage is a pivotal factor of consecutive parenchymal damage in the brain, in particular in delayed injuries (11).

X-ray damage to human tissues can be found in three systems: parenchyma or epithelia, stromal elements, and blood vessels (1, 2). In the parenchyma, the predominant findings are atrophy, metaplasia, cellular atypia, dysplasia or neoplasia (1, 2). In an earlier report on radiation damage with technical application of this irradiation model, our group described the atrophy and metaplasia of organs closely related to the nerve, e.g. the major salivary glands (28). Furthermore, neoplasms arising inside the irradiation field in this species were diagnosed and immunohistochemically classified (31), providing evidence of different entities arising inside and outside of the irradiation field. In the facial nerves of this investigation, we found atrophic and metaplastic Schwann cells (not shown). Neither dysplasia nor neoplasia could be derived from the nerve sheath in these experiments. This study does not address irradiation-induced stromal changes in detail, e.g. alterations of fibroblasts or vessels, but it seems likely that stromal changes contribute to the alterations of the morphologic parameters. The importance of irradiation-induced vascular alterations is emphasised in the literature (13, 24).

Indeed, the nuclei of both facial nerves are closely located in the brainstem, and the nuclei of both sides were inside the irradiation field. It cannot entirely be ruled out that direct damage of the brainstem nuclei contributes to peripheral nerve damage. The evidence of the whole brainstem lying inside the irradiation field is a strong argument in favour of a direct damage of the cranial nerve, as shown by morphometric differences of nerve parameters in nerves shielded or directly exposed to the source of the same individual. Therefore, the intra-individual (side-related) differences between nerve fibres located inside or outside the irradiation field found in this morphological study are considered to be caused by the interaction of X-rays and the nerve or the locally affected nutritive vessels or both.

**Consequences of scattering irradiation.** The ratio of axon to total area was significantly elevated in scattering irradiation-exposed nerve fibres explanted 7 to 9 months compared to nerves from untreated animals. In addition, the axon area was significantly greater in this group than in controls. Following these experimental results, it is reasonable to assume that similar conventional irradiation dosages result in relative and absolute increase of axon area compared to the total area. This increase is possibly due to the accumulation of degeneration products, thickening of neurofilaments and reduction of microtubuli (32, 33), since the expression of neurofilaments is accepted as determining the axon diameter (21).

In the early phase following irradiation, the morphometric values of the nerves show a different picture. In this period,
both axon area and total fibre area are diminished. The reduced ratio of axon to total area indicated a stronger loss of substance in axons than in Schwann cells.

These contradictory findings at different time points of the investigation are previously undisclosed and further studies should be undertaken.

The results of this study support the tentative conclusion that peripheral nerves are less radioresistant than currently accepted. Even small irradiation dosage causes measurable alterations of peripheral nerves. Schwann cells do not usually divide after forming the peripheral nerve sheath. After peripheral nerve damage Schwann cell mitotic activity is crucial for nerve repair (20). The loss of substance of both myelin and axon measured after the first months may be compensated by the regenerative capacity of the myelin sheath. However, this capacity is time dependent and differs in the peripheral nerve compartments.

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