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Post-irradiation bladder dysfunction: muscle strip findings

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Abstract Strips of rat detrusor muscle were studied in an organ bath 6 months after X-irradiation at doses of 15 and 25 Gy; cystometric studies in these animals had shown a persistent and significant reduction in compliance. The organ bath study demonstrated an increase in the purinergic sensitivity of irradiated detrusor muscle as compared with control. This was significant: $p < 0.0145$ for the 25 Gy dose group ($n = 8$) and $p < 0.0456$ for the 15 Gy group ($n = 8$) at an α,β-methylene-ATP concentration of 10^{-4} M (Mann-Whitney *U*-Test). There was no difference in sensitivity to cholinergic or noradrenergic stimulation, or to electrical stimulation of the transmural nerves. The finding of purinergic hypersensitivity in irradiated muscle, coupled with ultrastructural evidence of a neural injury, raises the interesting possibility that a denervation supersensitivity phenomenon may contribute to the pathophysiology of post-irradiation bladder dysfunction.

Key words Bladder dysfunction · Muscle strip · Rat · X-irradiation

Existing hypotheses for the late irradiation injury of the urinary bladder have concentrated on urothelial injury and fibrosis [6–8, 20, 21, 24], but such mechanisms would not account for the development of detrusor instability in up to a third of patients [14], and the potential functional significance of ultrastructural findings of radiation injury to smooth muscle and nerves [1, 2, 23] has been largely overlooked. The aim of this research was to assess rat detrusor muscle function in vitro following X-irradiation.

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Materials and methods

Initially a model for the late irradiation injury of the bladder was established using the female Wistar rat. These animals underwent localized X-irradiation of the bladder at doses of 15 or 25 Gy, in a single fraction under general anaesthesia. These doses were chosen as they are equivalent to the range of effective fractionated doses given to the bladder in radiotherapy of pelvic malignancies. Subsequently cystometry studies were performed regularly under enflurane anaesthesia, and the volume infused to produce a rise in intravesical pressure of 5 cm H_2O was taken as an index of complicance [23]. Once a sustained reduction in this compliance index had been demonstrated, the rats were killed by a blow to the head and cervical dislocation, and their bladders were removed for muscle strip study.

Muscle for organ bath study was taken from the lateral wall of each bladder and immediately placed in cold Krebs' solution (4 to 10°C). A mucosa-free strip of approximate size 5×2 mm was carefully prepared using a ×10 operating microscope, transferred to an organ bath and secured using 7/0 silk; a tension of 0.5 g was applied and a 1-h equilibration period was allowed prior to stimulation. One strip from each irradiated bladder was studied alongside a control; if any strip failed to generate a force of greater than 0.5 g on maximal contraction, it was discarded and replaced with another from the same bladder. The organ baths were perfused with Krebs' solution at 37.0–37.5°C, at a rate of 5 ml/min. Isometric contractions were recorded using resistive strain gauges (Hugo Sachs, K30 force transducers) connected via a bridge amplifier to a chart recorder.

Before embarking on studies comparing organ bath responses of irradiated and control muscle strips, it was necessary to establish various base-line characteristics for the normal Wistar rat bladder. Firstly, the electrical stimulus strength required to produce a maximal contractile response at a given frequency and pulse width was determined by varying voltage in the range 5–50 V. Secondly, to determine those electrical settings appropriate for producing nerve-induced contraction rather than direct muscle stimulation, pulse width was varied in the range 0.1–1 ms in the absence and in the presence of tetrodotoxin 10^{-9} M. This abolishes nerve-mediated responses by blocking the action potential. Finally, muscle strips were stimulated using various means to establish the most reliable method of generating a reproducible maximal response from a given strip. Stimuli used included electrical stimulation at various settings. high-dose acetylcholine (10^{-4} M) and high-dose potassium. Determining the maximal contraction that a strip can generate is important as it permits all other responses of that strip to be expressed as a percentage of this maximum. Absolute contractile force values cannot be used for comparison of different strips as all will contain a different number of contractile units.

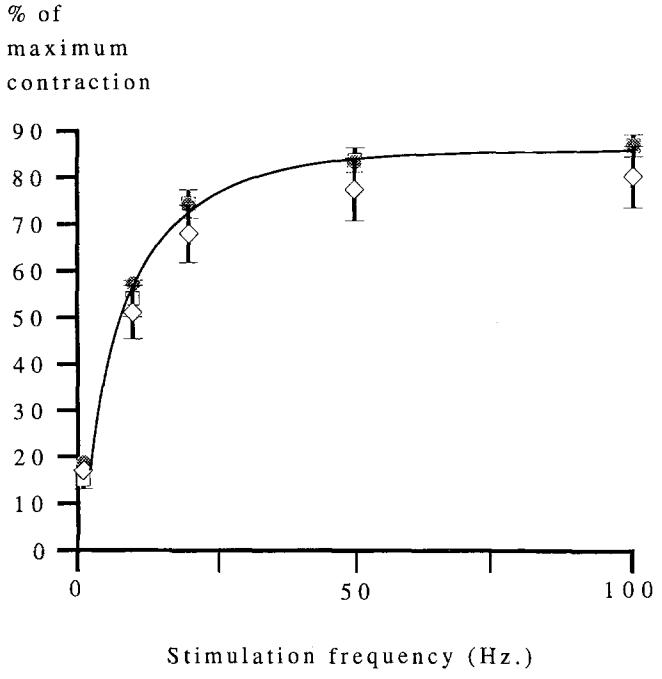

Fig. 1 Frequency-response curves for electrical stimulation of transmural nerves (20 V, pulse width 0.1 ms): both dose groups. *Points* are the mean ± SEM; □ control ($n = 10$); ▩ 15 Gy ($n = 8$); ◇ 25 Gy ($n = 8$)

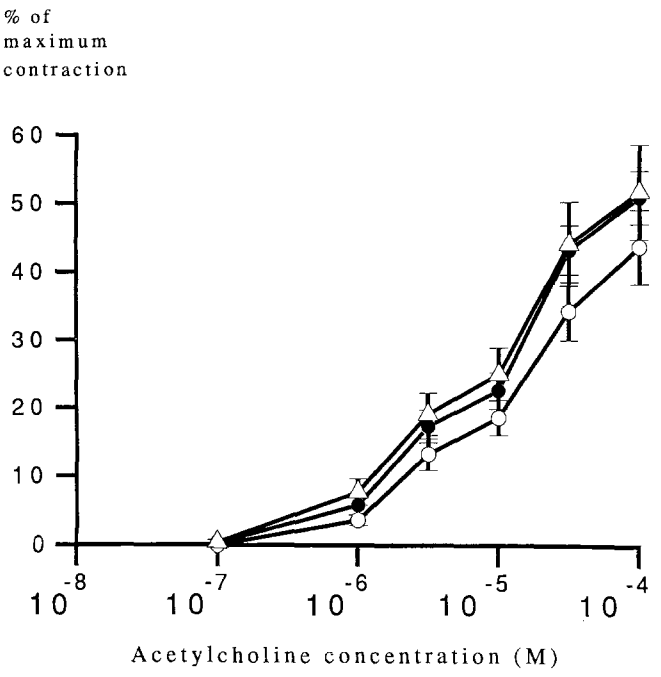

Fig. 2 Acetylcholine sensitivity: both dose groups. *Points* are the mean ± SEM; △ 25 Gy ($n = 9$); ● control ($n = 14$); ○ 15 Gy ($n = 9$)

To test the hypothesis that there may be changes in nerve-mediated mechanisms of bladder muscle activation following X-irradiation, strips of irradiated (15-Gy and 25-Gy dose groups) and control muscle were set up in adjoining organ baths. After a 1-h equilibration period, each strip was stimulated at a voltage of 20 V, frequency 50 Hz and pulse width 1 ms. Preliminary studies in control strips (above) had demonstrated that these settings were the most reliable means of provoking a reproducible maximal response from a given muscle strip. Stimulation at these electrical settings was repeated regularly throughout each experiment to ensure that there was no significant decline in the responsiveness of any individual muscle preparation. To produce frequency-response curves for nerve-induced contraction, electrical stimulation was performed at a maximal stimulus strength of 20 V, pulse width 0.1 ms (as determined by stimulation in the presence of tetrodotoxin, above), and frequency range 1–100 Hz. Each stimulus train was applied for 5 s with a resting period of 2 min before repetition. Stimulation was then performed successively using acetylcholine, noradrenaline and α,β-methylene ATP, as these are all known to have transmitter function within the rat bladder. Cholinergic stimulation was achieved using acetylcholine chloride (Sigma) in the concentration range 10^{-8}–10^{-4} M, and added to the perfusate. The lower concentrations were used initially, and stimulation was stopped as soon as the response had reached a maximum – usually after 30–120 s of perfusion. A resting period (perfusion with standard Krebs' solution) of at least 5 min was allowed before increasing the acetylcholine concentration, to allow full recovery to occur and to ensure a thorough wash-out of the previous additive. Noradrenergic and purinergic stimulation were performed in a similar fashion using (–)-Arterenol bitartrate 10^{-8}–10^{-4} M (noradrenaline, Sigma) and α,β-methylene-ATP 10^{-8}–10^{-4} M (Sigma) respectively. With the latter, special care was taken to stop stimulation as soon as the contractile response had reached a peak; prolonged exposure to this ATP analogue causes desensitization at P_2 purinoceptors [10]. At the end of the study, neural stimulation (20 V, pulse width 0.1 ms, frequency 1–50 Hz) was repeated in the presence of atropine sulphate 10^{-5} M added to the perfusate, to determine the degree of atropine resistance on neural stimulation of irradiated muscle. This intervention was performed last as atropine produces a prolonged depression of smooth muscle responsiveness.

The order of all interventions was standardized for all strips. All statistical analyses were performed using the Mann-Whitney *U*-test for non-parametric distributions.

Results

Rats which had received either 15 or 25 Gy showed a biphasic reduction in the compliance index when compared with controls [23]. The first reduction occurred at about 4 weeks post-irradiation and was by as much as 34% in the 25 Gy dose group. After transient recovery there was a second irreversible reduction starting at 3–4 months; the compliance index was reduced by as much as 48% by 6 months. The cystometry experiment was terminated at this time, and smooth muscle was taken for organ bath study.

With respect to the determination of in vitro base-line characteristics of the Wistar rat bladder, it was found that a stimulus strength of 20 V was sufficient to generate a maximal electrical response for a given frequency of stimulation and pulse width. Stimulation at a pulse-width of 0.1 ms was almost entirely prevented by tetrodotoxin (10^{-9} M), confirming that the muscle response at this setting was dependent upon nerve function. Direct electrical stimulation at 20 V, 50 Hz and pulse width 1 ms was found to be the most reliable means of provoking a maximal response. Thus for the comparison of all further

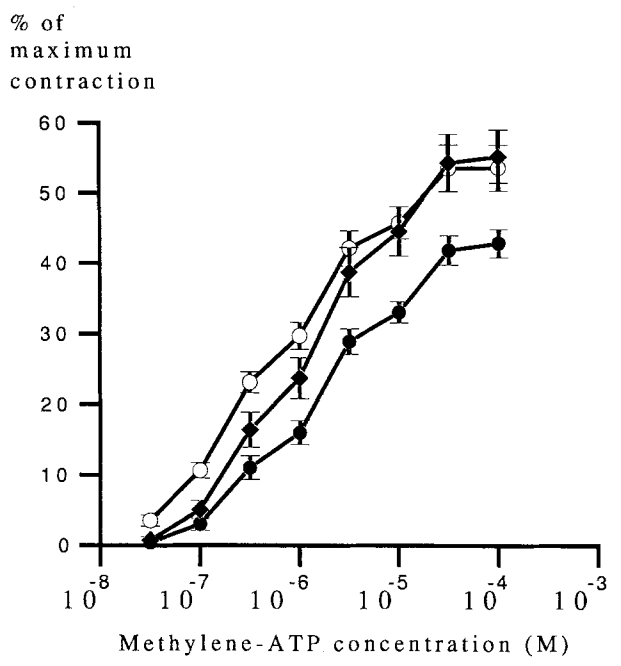

Fig. 3 Purinergic sensitivity: both dose groups. *Points* are the mean ± SEM; ○ 15 Gy ($n=8$); ◆ 25 Gy ($n=8$); ● control ($n=10$)

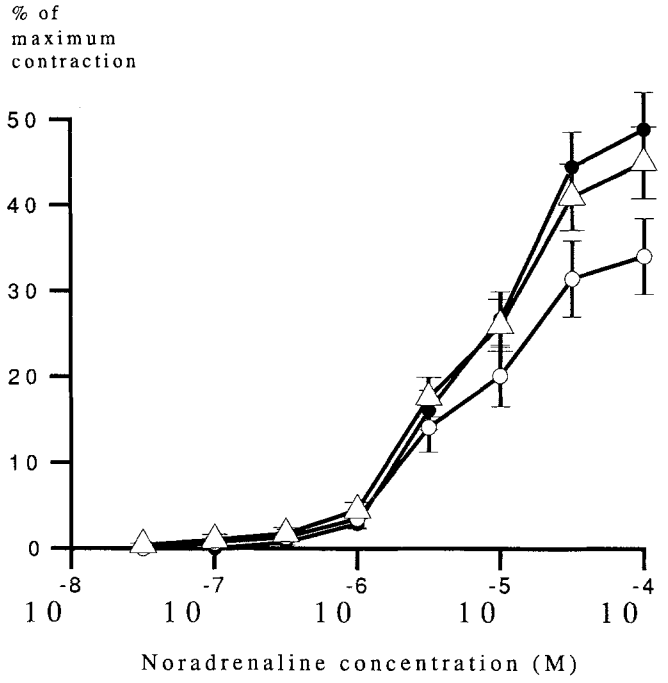

Fig. 4 Noradrenaline sensitivity: both dose groups. *Points* are the mean ± SEM; ● control ($n=22$); △ 25 Gy ($n=17$); ○ 15 Gy ($n=17$)

results, contractions of an individual muscle strip provoked by any stimulus were expressed as a percentage of the response of that strip to electrical stimulation at these settings (20 V, 50 Hz, 1 ms).

To assess whether or not there were changes in nerve-induced contraction following irradiation, frequency-response curves were constructed for electrical stimulation at 20 V and a pulse width of 0.1 ms (Fig. 1). There was no significant difference between irradiated strips ($n=8$, 25 Gy; $n=8$, 15 Gy) and control bladder strips ($n=10$), and no difference in the magnitude of the atropine-resistant component; this varied from 60% at a stimulation frequency of 50 Hz to almost total atropine resistance at 1 Hz in all strips. Likewise there was no difference between the sensitivity of irradiated muscle ($n=9$, 25 Gy; $n=9$, 15 Gy) and control muscle ($n=14$) to acetylcholine (Fig. 2), which is the main motor transmitter of the bladder. However, muscle strips from rats in both irradiation dose groups demonstrated a significant increase in sensitivity to α,β-methylene-ATP as compared with control muscle strips ($n=10$) (Fig. 3). At a methylene-ATP concentration of 10^{-4} M, this was significant at $p<0.0145$ for the 25 Gy dose group ($n=8$) and $p<0.0456$ for the 15 Gy group ($n=8$).

Although noradrenergic pathways are probably of only limited significance in the body of the bladder, the sensitivity of irradiated muscle strips to noradrenaline was assessed. There was no difference between strips from either the 25 Gy dose groups ($n=17$) or the 15 Gy dose group ($n=17$) and control rats ($n=22$) when stimulated using noradrenaline (Fig. 4).

In all graphs, the values plotted are the mean and standard error of individual muscle strip responses expressed as a percentage of the maximal response shown by each strip.

Discussion

There have been few studies of irradiated bladder smooth muscle function in vitro. Michailov et al. [13] have reported an acute reduction in the sensitivity of irradiated detrusor muscle to acetylcholine after X-irradiation. However, only muscle strips which had been irradiated with a massive dose of 50 Gy were studied, and none later than 35 days after irradiation. Thus the timing of these changes corresponded to the self-limiting acute radiation cystitis reaction. In addition threshold acetylcholine doses were measured as an index of sensitivity, down to concentrations as low as 10^{-10} M: contractile responses are very difficult to quantify at such low concentrations, especially after allowing for spontaneous muscle activity.

This study is the first to report changes in smooth muscle function at the time of late irradiation injury, and a significant increase in sensitivity to purinergic stimulation has been demonstrated. This is very interesting when considered in the light of ultrastructural evidence that focal nerve injury may occur post-irradiation [1, 2, 23]. When autonomic effector organs become denervated they may become increasingly sensitive to transmitters and chemical agents [5]. Two types of this denervation supersensitivity have been recognized [22]. The first is an

increase in sensitivity to a particular neurotransmitter type, and may be due to accumulation of this agonist at receptor sites secondary to loss of the normal mechanisms for its elimination; this has been labelled as "deviation supersensitivity" [25]. The second type of supersensitivity occurs as a result of some alteration in the physiology of smooth muscle cells, and a number of mechanisms have been proposed for this "non-deviation supersensitivity", which is usually non-specific [25].

The phenomenon of denervation supersensitivity has been reported in bladders which have been denervated surgically. Sethia et al. [16] found that following circumferential supratrigonal bladder transection, minipigs developed cholinergic supersensitivity and detrusor instability. Similar changes in cholinergic sensitivity have also been reported in cases of detrusor instability developing secondary to bladder outflow obstruction [9, 17, 19], and on the basis of immunohistochemical studies it was suggested that this may be due to a loss of acetylcholine-containing nerve fibres. The increase in purinergic sensitivity demonstrated in this research may represent an equivalent denervation supersensitivity phenomenon affecting purinergic activation mechanisms. The importance of ATP as a neurotransmitter in the rat is proven [3, 4], and it may be that a reduction in local ATP release, secondary to a loss of small unmyelinated terminal nerve fibres following X-irradiation, may lead to up-regulation of P_2 purinoceptors. This is not without precedent: an increase in the concentration of cardiac α- and β-adrenoceptors has been reported 200 and 400 days after local X-irradiation of the rat heart [12, 15]. It was suggested that this up-regulation was initiated by a decreased sympathetic output of cardiac nerves, due either to a direct effect of the radiation on terminal nerves or secondary to X-ray-induced capillary damage and local hypoxia. Purinergic receptor up-regulation in the bladder would explain the specificity of the change in sensitivity documented in this research.

If there is a loss of ATP-releasing nerve fibres, it might seem paradoxical that there was no change in the frequency-response characteristics of the irradiated muscle on transmural nerve stimulation. However, although low-frequency stimulation of transmural nerves causes contraction by almost entirely atropine-resistant mechanisms [4], these may involve transmitters other than ATP. Finally, the question arises as to whether or not a similar pathophysiological change could explain post-radiotherapy bladder dysfunction in patients. Sjögren et al. [18] have found that although there is little atropine resistance to electrical stimulation in the normal human bladder, strips of muscle from patients with cystometrically verified instability demonstrated degrees of atropine resistance as high as 50%. Thus disease processes may lead to changes in detrusor activation mechanisms, and any alteration in the intricate balance of neurotransmitters and neuromodulators could be of profound functional importance [11].

This research has raised interesting questions about the pathophysiological basis of bladder dysfunction developing late after pelvic irradiation. The ultrastructural evidence of neural damage, together with the organ bath finding of purinergic supersensitivity, suggest that neural mechanisms may be of functional significance. This is worthy of further investigation.

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