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ORIGINAL ARTICLE

Comparison of subcutaneous and intraperitoneal injection of D-luciferin for in vivo bioluminescence imaging

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Abstract
Purpose We compared subcutaneous (SC) injection and intraperitoneal (IP) injection of D-luciferin for in vivo bioluminescence imaging (BLI) to determine the utility of SC injection.
Methods Mice bearing SC tumours stably expressing firefly luciferase underwent in vivo BLI using SC and IP injection of D-luciferin. BLI studies were repeated at an interval of 3 h using a given injection route to assess repeatability and using different injection routes to assess correlation. In mice bearing both SC and IP tumours, BLI was performed successively using intravenous (IV), SC, and IP injection of D-luciferin. Haematological malignancy model mice underwent BLI using SC and IP injection.
Results In SC tumours, the peak time was slightly shorter and the peak signal was greater using SC injection than using IP injection. The repeatability of determining peak signals was comparable between the two injection routes, and a good correlation was observed between them. In mice bearing both SC and IP tumours, signals from IP tumours relative to those from SC tumours were much greater using IP injection than using IV or SC injection. In the haematological malignancy model, signals from the spleen relative to those from the bone marrow were greater using IP injection than using SC injection.
Conclusion In addition to rare injection failure, the IP injection of D-luciferin led to the overestimation of signals from IP tissues. For BLI, SC injection was shown to be a convenient alternative to IP injection.

Keywords Bioluminescence imaging · Luciferase · Mouse · Intraperitoneal injection · Subcutaneous injection

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Introduction

In vivo bioluminescence imaging (BLI) enables the evaluation of the magnitude and distribution of luciferase gene expression in intact laboratory animals and can be used for various purposes such as monitoring tumour [1, 2], studying cell trafficking [3, 4], researching infectious diseases [5], monitoring gene therapy [6], investigating transcriptional regulation [7], and evaluating protein-protein interactions [8]. Typically, D-luciferin is injected into animals expressing firefly luciferase for in vivo BLI and is oxidized by luciferase, resulting in light emission. The bioluminescence signals are detected using a charge-coupled device (CCD) camera, which visualizes luciferase activity in the animals.

The substrate D-luciferin can be administered to animals using intraperitoneal (IP) or intravenous (IV) injection [9], and IP injection is generally preferred because of its convenience. After IP injection, D-luciferin is absorbed through the peritoneum and reaches luciferase-expressing

tissues via the bloodstream. However, IP injection of D-luciferin produces unexpectedly weak signals on rare occasions owing to injection into the bowel [10, 11]. Variations in the absorption rate through the peritoneum may distort the reproducibility of signal quantification [12]. A biodistribution study of radioiodine-labelled D-luciferin demonstrated higher uptake in the gastrointestinal organs, pancreas, and spleen after IP injection than after IV injection [13]. Although in vivo BLI using IP injection has been used for the evaluation of IP tumours [14–17], direct diffusion, other than delivery via the systemic circulation, may cause preferential distribution of D-luciferin in IP tumours, leading to the overestimation of luciferase activity in IP tumours relative to that in extraabdominal tumours. The validity of IP injection as the route of administration for D-luciferin remains to be evaluated.

D-Luciferin was administered subcutaneously for in vivo BLI in some previous studies [18–20]. Positive correlation between luminescence intensity and tumour volume was found in a rat model of brain tumour using subcutaneous (SC) injection [18]. Continuous SC infusion of D-luciferin to mice bearing SC tumours has enabled the effects of drugs on BLI signals to be assessed [19, 20]. These studies demonstrated that D-luciferin given subcutaneously can be delivered to brain tumours and SC tumours. SC injection is another easy route of administration and has potential as an alternative to IP injection. In the present study, we performed in vivo BLI using SC and IP injection of D-luciferin in tumour model animals. The intensity and temporal pattern of BLI signals were investigated in a SC tumour model. Moreover, in animals bearing lesions both inside and outside the peritoneal cavity, the distribution of BLI signals was compared between different injection routes. This study aimed to investigate the effect of the injection route on the results of in vivo BLI and to determine the utility of SC injection as a method of D-luciferin administration.

Materials and methods

Cell lines

The interleukin-3-dependent murine pro-B cell line Ba/F3 was cotransfected with the firefly luciferase gene and the wild-type p190 BCR-ABL fusion gene using a previously described retroviral method [21]. The established cells were named Ba/F3-Luc/Wt cells and were maintained in RPMI-1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and 1% penicillin/streptomycin (Invitrogen) in the absence of interleukin-3. Firefly luciferase is stably expressed under the control of the long terminal repeat of Moloney murine leukaemia virus in the cells. The p190 BCR-ABL gene is important in the development of acute lymphoblastic leukaemia [22] and causes factor-independent, autonomous proliferation when transformed into Ba/F3 cells [23]. The human colon cancer cell line HCT116 was transfected with the firefly luciferase gene using the retroviral method, leading to the establishment of HCT116-Luc cells. They were maintained in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/streptomycin. Cell cultures were incubated at 37°C in an atmosphere containing 5% CO_2.

Animals

Female BALB/c nu/nu mice at 8 weeks of age were inoculated with the luciferase-expressing cells and underwent in vivo BLI at the various times specified for each experiment. The mice were obtained from SLC Japan (Hamamatsu, Japan) and were handled in accordance with the guidelines of the Institute of Medical Science, University of Tokyo. The experiments were approved by the Committee for Animal Research at the institution.

SC tumour model

Mice bearing SC tumours underwent in vivo BLI repeatedly with SC and IP injection of D-luciferin. Mice were inoculated subcutaneously in the dorsal flank with 5×10^5 HCT116-Luc cells, mixed with Matrigel (BD Biosciences, San Jose, CA). The flow chart of the BLI studies is presented in Fig. 1. In vivo BLI was performed 8 days after inoculation using SC injection of D-luciferin, and mice were divided into two groups (groups A and B, $n=6$ each) having comparable signals. The results of day-8 imaging were not used for further analysis. On day 9, the mice in group A were examined twice at an interval of 3 h using SC injection of D-luciferin to assess the repeatability of determining peak signals. They underwent BLI using SC injection 24 h after the first day-9 imaging, followed 3 h later by BLI using IP injection, to evaluate the correlation between peak signals determined using the two injection routes. On day 13, the mice in group A were examined twice at an interval of 3 h, using IP injection. They underwent BLI using IP injection 24 h after the first day-13 imaging, followed 3 h later by BLI using SC injection. The mice in group B were imaged using IP and SC injections when the mice in group A were imaged using SC and IP injections, respectively. This schedule was determined to achieve comparable tumour burdens in the animals examined using SC and IP injections, while minimizing the number of animals required for the study.

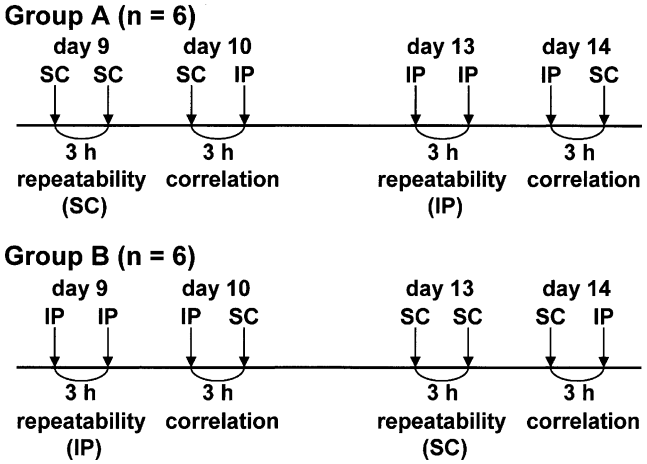

Fig. 1 Flow chart of the experiments in the SC tumour model. *SC* and *IP* indicate BLI using SC and IP injection, respectively. On day 9, the mice in group A were examined twice at an interval of 3 h using SC injection of D-luciferin to assess the repeatability. They underwent BLI using SC injection on day 10, followed 3 h later by BLI using IP injection, to evaluate the correlation between the two injection routes. On day 13, the mice in group A were examined twice using IP injection. They underwent BLI using IP injection and, subsequently, using SC injection on day 14. The mice in group B were imaged using IP and SC injections when the mice in group A were imaged using SC and IP injections, respectively

In vivo BLI was performed using a cooled CCD camera system (IVIS Imaging System 100; Xenogen, Alameda, CA). Mice were injected with 75 mg/kg D-luciferin (Beetle Luciferin Potassium Salt, Promega, Madison, WI) in 100 μl of phosphate-buffered saline subcutaneously near the scapula or intraperitoneally, and were placed in the light-tight chamber of the CCD camera system under isoflurane anaesthesia. Beginning 4 min after injection, dorsal luminescent images with an exposure time of 1 s were acquired sequentially at a rate of one image per minute up to 20 min and 24 min after SC and IP injections, respectively. During the interval between successive BLI studies, mice were returned to the cage and received food and water ad libitum. No apparent effect of the procedure on the health of the mice was noticed.

An elliptical region of interest (ROI) was placed over the SC tumour, and the total signal in the ROI (photons per second) was quantified using Living Image software (version 2.50; Xenogen). The same ROI was applied to all images acquired sequentially in a single imaging session for a given mouse. The total signal intensity was plotted against time after D-luciferin injection to generate a time–intensity curve. Peak time and peak signal were determined, and the peak signal was regarded as an indicator of tumour burden. Prior to statistical testing, the peak signal was transformed logarithmically. The peak time and peak signal were compared between SC and IP injections using an unpaired *t* test with all data and using a paired *t* test with paired data acquired on the same day with alternating injection routes only. The repeatability of peak signal determination was assessed from paired data acquired on the same day using a given injection route. The difference in the peak signal between the first and second measurements was plotted against the average. The bias was defined as the mean of the differences, and the 95% confidence interval was the range of ±1.96 SD of the mean. The correlation between peak signals determined using the two injection routes was evaluated from paired data acquired on the same day with alternating injection routes, and linear regression analysis was performed by the least squares method.

Model of coexisting SC and IP tumours

In mice bearing both SC and IP tumours, BLI was performed successively using IV, SC, and IP injection of D-luciferin, and the distribution and time-course of the BLI signals were compared between injection routes. Eight mice were inoculated subcutaneously near the upper border of the sternum with 5×10^5 HCT116-Luc cells and intraperitoneally with 1×10^6 HCT116-Luc cells. The cells were mixed with Matrigel before inoculation. Ten or 11 days after inoculation, each mouse underwent three BLI studies using IV, SC, and IP injection of D-luciferin on a single day. First, mice were injected with 15 mg/kg D-luciferin (100 μl) intravenously into the tail vein, and a 1-s ventral luminescent image was acquired every minute from 1 to 40 min after injection. Three hours after IV injection, 75 mg/kg D-luciferin (100 μl) was injected subcutaneously near the scapula, and the same data acquisition was performed. Three hours later, 75 mg/kg D-luciferin (100 μl) was injected intraperitoneally, followed by the same data acquisition. The injection dose of D-luciferin was reduced for IV injection because the same dose has been shown to produce much greater signal after IV injection than after IP injection in SC tumours [12].

An elliptical ROI was drawn on the chest over the SC tumour, and a rectangular ROI was set over the entire abdomen. The total signal intensities in the ROIs (photons per second) were plotted against time, and the area under the curve (AUC) during the 40-min acquisition period was determined for each ROI. The relative abdominal signal was defined as the ratio of the AUC for the abdominal ROI to that for the chest ROI, and was compared between injection routes after logarithmic transformation by one-way repeated measures analysis of variance followed by post hoc analysis using Fisher's least significant difference test. The relative abdominal signal was also calculated using the peak signal instead of the AUC and analysed similarly.

Haematological malignancy model

Haematological malignancy model mice underwent in vivo BLI using SC and IP injection of D-luciferin. Four mice were inoculated with 2×10^6 Ba/F3-Luc/Wt cells intravenously via the tail vein. In this model, the inoculated cells have been shown to proliferate mainly in the bone marrow, spleen, and liver [24]. In vivo BLI was performed 16 days later. The mice received 75 mg/kg D-luciferin intraperitoneally and, 3 h later, subcutaneously near the scapula. A left-lateral luminescent image with an exposure time of 5 s was acquired every minute from 1 to 30 min after each injection.

Elliptical and circular ROIs were applied within the spleen and left knee, avoiding the influence of signals from neighbouring structures. The sizes of the ROIs were fixed for all mice and for both injection routes. Average signal intensity (photons per second per square centimetre per steradian) was determined to produce a time–intensity curve, and the AUC during the 30-min acquisition period was determined for each ROI. The relative spleen signal was defined as the ratio of the AUC for the spleen ROI to that for the knee ROI and was compared between SC and IP injections using a paired *t*-test. In addition, the relative spleen signal was determined using the peak signal.

Statistical analysis

Data are presented as means±SD. Statistical testing was performed as described in each section, after the assessment for normal distribution with the Kolmogorov-Smirnov test. *P* values <0.05 were considered statistically significant.

Results

SC tumour model

In the SC tumour model, a total of 96 BLI studies, 48 each for SC and IP injections, were performed. Essentially no signal was demonstrated in one study using IP injection, presumably because of an intrabowel injection. The data from this failed study and also from the study of the mouse performed on the same day using SC injection were excluded from the analysis. Consequently, 94 studies, 47 each for SC and IP injections, were analysed. The repeatability was assessed using 12 pairs each of BLI data for SC and IP injections. The correlation between the two routes was evaluated using 23 pairs. A luminescent image was acquired just before the second BLI on a given day; the signal in the preinjection imaging was less than 1% of the peak signal in the second BLI for all experiments and was ignored in the analysis.

When all data were used for analysis, the peak time using SC injection (9.6±1.8 min, range 6–14 min) was slightly but significantly shorter than that using IP injection (11.6±2.1 min, range 7–16 min; $p<0.0001$). The peak signal was significantly greater for SC injection ($2.13\times10^9 \pm 1.53\times10^9$ photons/s) than for IP injection ($1.43\times10^9 \pm 1.00\times10^9$ photons/s, $p<0.05$). When the mean peak time for the respective injection route (10 and 12 min for SC and IP injection, respectively), instead of the peak time for each measurement, was used to determine the signal intensity representing the tumour burden, the signal was underestimated by 2.6±3.4% (maximum 15.1%) or 2.9±4.0% (maximum 19.0%) for SC or IP injection, respectively ($p>0.05$, Mann-Whitney *U* test). The repeatability of the peak signal determination was similar between SC and IP injections (Fig. 2). The analysis of repeatability for SC injection estimated the bias as −0.002 with a 95% confidence interval of −0.198 to 0.195. For IP injection, the bias was estimated as −0.028 with a 95% confidence interval of −0.243 to 0.187. When paired data acquired on the same day with alternating injection routes were exclusively used for analysis, the peak time was slightly shorter (SC 9.8±2.0 min, IP 11.6±1.7 min; $p<0.0001$) and the peak signal was greater for SC injection than for IP injection (SC $2.23\times10^9 \pm 1.4\times10^9$ photons/s, IP $1.61\times10^9 \pm 1.09\times10^9$ photons/s; $p<0.0001$). The peak signal

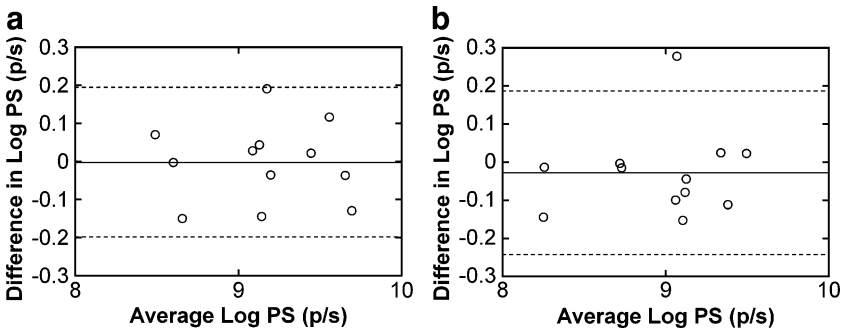

Fig. 2 Repeatability of peak signal (*PS*) determinations using SC (**a**) or IP (**b**) injection in the SC tumour model. The difference between repeated measurements was plotted against the average of the measurements after logarithmic transformation. The *solid line* represents the mean of the differences (bias), and the *broken lines* represent the mean±1.96 SD (95% confidence interval)

using SC injection was closely correlated with that using IP injection (Fig. 3, r=0.890, p<0.0001).

Model of coexisting SC and IP tumours

In mice bearing both SC and IP tumours, one study using the IP injection of D-luciferin showed unreasonably weak signals 10 days after cell inoculation. The entire series of BLI studies were performed again for the mouse on the following day, and the results of the retry were used for analysis. In vivo BLI using IV, SC, and IP injections showed definitely different distributions (Fig. 4) and time-courses (Fig. 5) of BLI signals. Although the abdominal signal from the IP tumour was weak relative to the chest signal from the SC tumour after IV or SC injection of D-luciferin, it was strong after IP injection. The relative abdominal signal using IP injection (10.48±8.75) was more than 30 times that using IV (0.30±0.30) or SC (0.28±0.24) injection when the AUC was used for the calculation. The relative abdominal signal differed significantly between injection routes (p<0.0001), and post hoc analysis revealed significant differences between IV and IP injection and between SC and IP injection (p<0.0001 for both). The relative abdominal signal determined from the peak signal was 0.22±0.40, 0.22±0.22, and 7.58±6.95 for IV, SC, and IP injections, respectively. It differed significantly between injection routes (p<0.0001), and significant differences were found between IV and IP injection and between SC and IP injection (p<0.0001 for both). With regard to the time-course, the IV injection of D-luciferin caused maximal luminescence immediately, followed by a rapid decline, for both the SC and IP tumours, except that signals from IP tumours in two mice increased gradually for 5 or 10 min. All curves obtained using SC injection showed a single peak, representing a gradual increase followed by a gradual decrease. The peak time for the IP tumour (15.1±4.7 min) was significantly longer than that for the SC tumour (9.9±0.6 min, p<0.05, paired t test). After IP injection, the curve pattern for the SC tumour was similar to that after SC injection, and the peak time was 10.0±0.5 min. However, strong BLI signals were observed for the IP tumours immediately after IP injection. Thereafter, the abdominal signals did not change largely in two mice. In the other six mice, they decreased initially, then increased gradually, and remained strong up to the end of the imaging period.

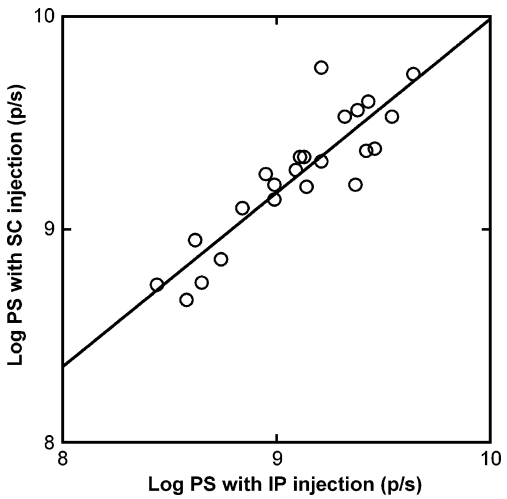

Fig. 3 Correlation of peak signal (*PS*) between IP and SC injections in the SC tumour model. The solid line represents regression line (y = 0.818x + 1.81; r=0.890; p<0.0001) that was determined after logarithmic transformation

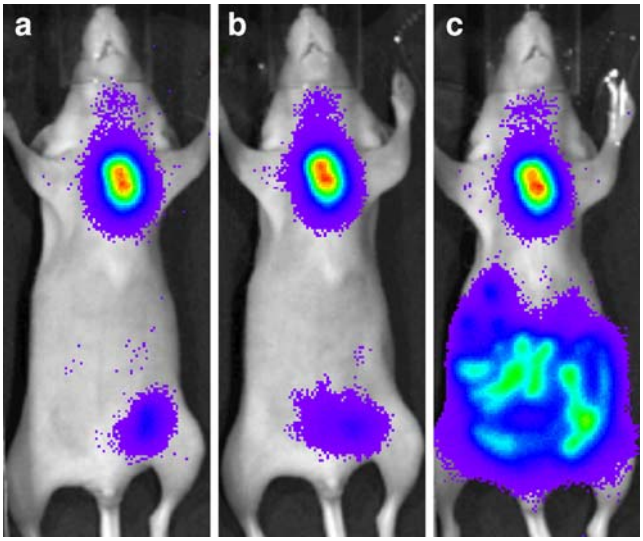

Fig. 4 Ventral BLI images obtained after IV (**a**), SC (**b**) or IP (**c**) injection of D-luciferin. The mouse was inoculated with HCT116-Luc cells subcutaneously near the upper border of the sternum and intraperitoneally. The pseudocolour luminescent images (blue, green, yellow, and red from least to most intense) are overlaid on the grey-scale photographic images. The upper level of the colour scale was adjusted for each panel so as to similarly display the SC tumour, and the lower level was set at 0.5% of the upper level

Haematological malignancy model

In mice inoculated intravenously with Ba/F3-Luc/Wt cells, in vivo BLI in the left-lateral projection showed signals suggestive of the involvement of the spleen and bone marrow including the left knee. The visualization of the spleen was more prominent after IP injection of D-luciferin than after SC injection (Fig. 6). When the AUC was used for analysis, the relative spleen signal using IP injection (2.66±0.68) was about three times that using SC injection (0.78±0.26), and the difference was statistically significant (p<0.05). The relative spleen signal calculated from the peak signal differed significantly between SC and IP injections (SC 0.80±0.28, IP 3.30±1.07; p<0.05). Differ-

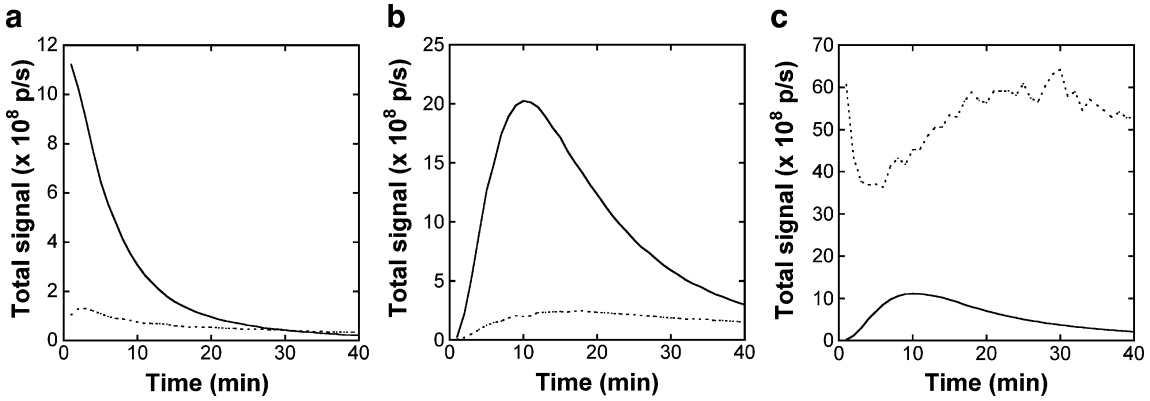

Fig. 5 Examples of the time–intensity curves determined after IV (**a**), SC (**b**) or IP (**c**) injection of D-luciferin in a mouse bearing both SC and IP tumours (the same mouse as that presented in Fig. 4). The *solid* and *broken lines* are curves for the SC and IP tumours, respectively

ences between the two injection routes were also observed in the time-course of BLI signals (Fig. 7). After SC injection, the shape of the time–intensity curve was similar between the spleen and knee, and a clear single peak was observed in all curves. The peak time was similar between the spleen (8.8±1.0 min) and knee (8.0±1.4 min). Strong luminescence from the spleen was observed immediately after IP injection in all four mice. Thereafter, the spleen signal gradually declined in two mice, remained approximately constant for about 10 min and then decreased in one mouse, and peaked at 13 min in the other mouse. The knee curve after IP injection showed a single peak, similarly to that after SC injection, and the peak time was 11.8± 1.5 min.

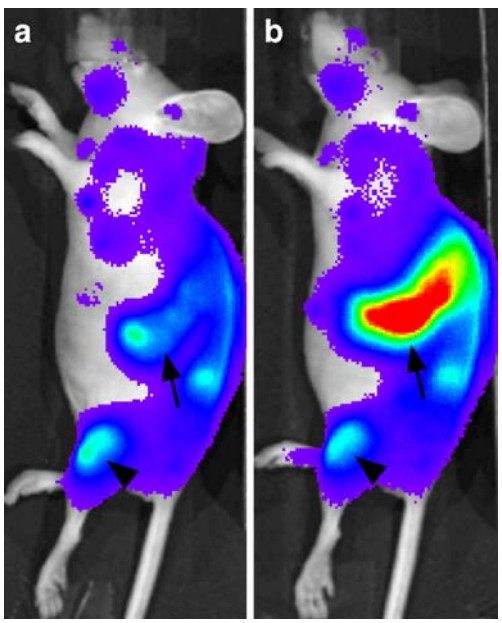

Fig. 6 Left-lateral BLI images obtained after SC (**a**) or IP (**b**) injection of D-luciferin. The mouse was inoculated intravenously with Ba/F3-Luc/Wt cells. BLI signals suggestive of cell proliferation in the spleen (*arrows*) and bone marrow, including the left knee (*arrowheads*) are observed, and the splenic signal is more evident after IP injection. The upper level of the colour scale was adjusted for each panel so as to similarly display the bone marrow lesions, and the lower level was set at 2% of the upper level

Discussion

Although most researchers use IP injection to administer D-luciferin to animals for in vivo BLI, it may rarely result in intrabowel injection and a consequent lack of substantial luminescence. In the present study, 2 of 61 IP injections (3.3%) produced negligible signals, indicating injection failure, and the resulting BLI data were excluded from the analysis. The rate of IP injection failure has been reported to be about 4% [10], less than 10% [11], and 2.9% [25], similar to the results in the present study. The failure rate is low; however, injection failure may occur in many mice at one or more time point(s), reducing the number of complete datasets when BLI is repeated in individual mice for longitudinal assessments. Although BLI may be performed after additional D-luciferin injection in the event of unexpectedly weak luminescence, it is not necessarily easy to determine whether the cause of a weak signal is a low level of luciferase expression or injection failure. The possibility of partial intrabowel injection with partial successful IP injection poses a more complicated problem. In contrast, the lack of a substantial signal, suggestive of injection failure, was not seen after the SC injection of D-luciferin in the present study. The absent risk of injection failure represents an advantage of SC injection over IP injection.

In mice bearing SC tumours, we measured BLI signals as a function of time after SC or IP injection of D-luciferin. Peak time was slightly shorter for SC injection. The peak signal obtained using SC injection was larger than and well

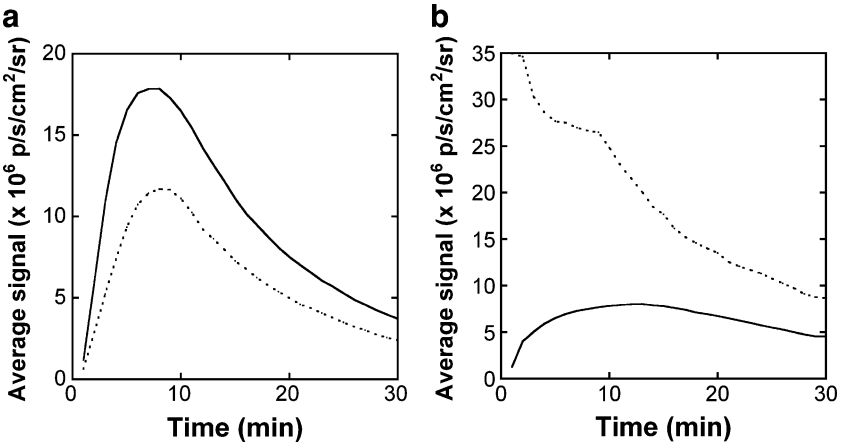

Fig. 7 Examples of the time–intensity curves determined after SC (**a**) or IP (**b**) injection of D-luciferin in an animal model of haematological malignancy (the same mouse as that presented in Fig. 6). The *solid* and *broken lines* are curves for the left knee and spleen, respectively

correlated with that obtained using IP injection. Repeatability was similar between the two injection routes. Overall, favourable absorption of D-luciferin injected subcutaneously is indicated, and SC injection appears to be comparable or superior to IP injection, even disregarding the possibility of injection failure. Although 12 mice received SC injection five times in 7 days in this series, local damage at the injection site was not observed, other than occasional minor SC haemorrhage, suggesting the safety of SC injection as an administration route for D-luciferin. The coefficients of variance of the peak signals in the SC tumour model were 71.8% and 70.4% for the SC and IP injections of D-luciferin, respectively. Although these values were derived from data obtained on different days after cell inoculation (days 9, 10, 13, and 14), large variability was observed even in data obtained on a given day. The variability appears to be ascribable primarily to variability in tumour growth itself and secondarily to variability in BLI measurement.

We used a coexisting SC and IP tumour model to determine whether IP injection causes preferential bioluminescence from IP tumours. The IP injection of D-luciferin markedly overestimated the IP tumours compared with IV injection as the standard method, indicating that special attention is required when using IP injection in animals having luciferase activity simultaneously inside and outside the peritoneal cavity. IP injection immediately caused strong bioluminescence from the IP tumours, although the signals from the SC tumours increased gradually. D-Luciferin is absorbed through the peritoneum and reaches SC tumours via the bloodstream. The time required for absorption and delivery causes a delay in luminescence after IP injection as compared with IV injection [12, 26]. The immediate luminescence from IP tumours after IP injection appears to be attributable to the direct arrival of D-luciferin. In most of the mice, the abdominal signals recovered after an initial reduction, and the recovery was too great to be explained by supply of D-luciferin through the bloodstream. D-Luciferin supplied through the bloodstream would produce smaller abdominal signals than those observed after SC injection; however, the recovery was much larger than expected. The direct arrival of D-luciferin from the peritoneal cavity to the IP tumour may follow a complicated time course.

In contrast to IP injection, the SC injection of D-luciferin provided a relative abdominal signal similar to that seen with IV injection, supporting the view that D-luciferin injected subcutaneously is delivered exclusively through the systemic circulation. SC injection is indicated to be superior to IP injection for assessing the whole-body distribution of luciferase expression in animals having luciferase inside and outside the peritoneal cavity. D-Luciferin diffuses around the injection site after SC injection, possibly leading to the overestimation of luciferase activity in the neighbouring tissues. In the present study, injection near the scapula did not cause an overestimation of the SC tumour near the upper border of the sternum. It is not clear how far a substantial amount of D-luciferin diffuses after SC injection, and selection of an injection site distant from the region to be evaluated is recommended.

We examined mice of a haematological malignancy model as a more realistic animal model bearing luciferase-expressing cells inside and outside the peritoneal cavity, and splenic signals relative to knee signals were determined after SC and IP injections of D-luciferin. Similar to the observations in IP tumours, IP injection caused early, preferential luminescence from the spleen. Although IV injection was not performed for this model, IP injection is indicated to cause overestimation of signals from the spleen. It appears that a substantial amount of D-luciferin can enter the spleen directly from the peritoneal cavity, as indicated in a biodistribution study of radioiodine-labelled

D-luciferin [13]. In the haematological malignancy model, we monitored disease progression [24] and response to total body irradiation [25] using in vivo BLI after IP D-luciferin injection, and demonstrated that whole-body BLI signals successfully reflect disease severity. In vivo BLI using IP injection is indicated to provide a useful index of whole-body tumour burden, despite overestimating the contribution of splenic involvement. BLI using SC injection may better reflect disease severity in this model, but this remains to be examined.

We repeated BLI at an interval of 3 h and ignored residual signals related to the preceding BLI studies in the analysis. Although we confirmed by preinjection imaging that residual signals were negligible for the SC tumour model, we did not perform preinjection imaging for the other models. For the model of coexisting SC and IP tumours, signals after IV injection declined rapidly, excluding major effects on subsequent BLI studies using SC injection. Residual signals related to SC injection do not appear to have influenced the results after IP injection substantially because signals from the SC tumour would have decreased sufficiently, as shown in the SC tumour model, and signals from the IP tumour were weak after SC injection. In the haematological malignancy model, preceding IP injection produced strong spleen signals, and signals measured after SC injection may have included some residual signals related to IP injection, especially for the spleen. Thus, overestimation of the spleen signal using IP injection may be more profound than that indicated by our results. Overall, ignoring residual signals related to the preceding BLI studies would not have had a major impact on our results suggesting that IP injection may cause overestimation of IP lesions.

We acquired multiple sequential images to determine the time-course of BLI signals after D-luciferin administration. In most BLI studies, image acquisition is performed at a single, predetermined time-point to assess the magnitude of luciferase expression. This strategy allows the imaging duration to be shortened and improves throughput of the measurements. In our experiments regarding the SC tumour model, the signal from the tumour did not differ markedly between the peak time determined in each sequential imaging and the mean peak time, justifying the assessment of tumour burden by single-point imaging at the mean peak time. This was true for both SC and IP injections and agreed with the results of previous studies using IP injection [10, 11].

In contrast, the time-courses of BLI signals from IP tumours after IP D-luciferin injection were complicated and markedly different from those of extraabdominal signals. The spleen curves were definitely different in shape from the bone marrow curves although the small number of mice studied prevented detailed assessment of the time-course of the spleen signal. Owing to the inconsistency in temporal patterns, the determination of appropriate imaging timing appears to be difficult, and imaging at multiple time-points is recommended in mice bearing luciferase-expressing cells inside and outside the peritoneal cavity when IP injection is adopted as the injection route. Future studies are needed to determine the best indicator: AUC, peak signal, or signal at a preindicated time-point. After SC injection of D-luciferin, the time-course of BLI signals was relatively consistent among regions and among mice, except for mildly delayed peak for the IP tumours, which appears to allow the assessment of tumour burden using single-point imaging or sequential imaging of a short duration even in models of extensive disease.

The assessment of tumour burden in animal models of disseminated peritoneal disease is a difficult task for conventional experimental methods and may be greatly aided by in vivo BLI [14, 16]. Our results indicated that the IP injection of D-luciferin would cause the overestimation of tumour burden inside the peritoneal cavity relative to that outside the peritoneal cavity and an inconsistency in the temporal patterns of the bioluminescent reaction. Nevertheless, the strong BLI signal from IP tumours offer high sensitivity and may be beneficial in animals suffering exclusively from disseminated peritoneal disease. It would be desirable to investigate the reproducibility and appropriate acquisition timing for the use of IP injection in such a model. In the present study, the injection dose of D-luciferin was smaller for IV injection than for SC and IP injection. If the dose were the same, IV injection would cause stronger bioluminescence than SC injection, with a simple, rapid time-course. IV injection in experienced hands can be recommended especially when the sensitivity of BLI using SC injection appears insufficient.

In conclusion, the IP injection of D-luciferin rarely fails to produce substantial signals and may cause the overestimation of luciferase activity inside the peritoneal cavity. SC injection is free from injection failure and offers consistent results for the luciferase-expressing tissues inside and outside the peritoneal cavity. No major drawbacks of SC injection were demonstrated in the present study, and SC injection was indicated to be a promising alternative to IP injection for in vivo BLI.

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Conflicts of interest None.

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